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APPLICATION NUMBER: ***60/555,320***
FILING DATE: ***March 23, 2004***

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Docket Number: 265/36

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53 (c).

INVENTOR(S)/APPLICANT(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
EPHRAIM	LANSKY	HAIFA, ISRAEL

☐ Additional inventors are being named on page 2 attached hereto

TITLE OF THE INVENTION (280 characters max)

SYNERGISTIC PROSTATE CANCER SUPPRESSION BY POMEGRANATE

CORRESPONDENCE ADDRESS

Direct all correspondence to:

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ENCLOSED APPLICATION PARTS (check all that apply)

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FILING FEE AMOUNT: $80 + 40

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government

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Respectfully submitted,

SIGNATURE _____ DATE 23 MAR 04

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USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

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P19LARGE/REV04

Preliminary Report on Evaluation of Pomegranate-based Products

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DATE: December 7, 2001

Contents

General comments
The studies described in this preliminary report were undertaken as an initial evaluation of certain products from Rimonest Inc. As such, they were done in good faith with the assumption that a suitable Sponsored Research Agreement could result from them if the data proved to be of interest. The data included in this report include a comparison of the relative cytotoxicity of punicic acid to the Rimonest products as well as an initial assessment of the relative ability of the materials to alter (i.e. inhibit) selected eicosanoid pathways.

In our opinion, the data clearly warrant further development and evaluation of these products. As of the date of this report, Rimonest Inc. has agreed to fund through a Sponsored Research Agreement (SRA) an initial safety (toxicology) study of two oil-based products in mice. This SRA is currently in preparation. A Confidentiality Agreement has been signed and returned to Rimonest Inc.

Description and analyses of materials
The company has provided MDACC with a cold-pressed or pure pomegranate seed oil (PPSE) and an enhanced preparation consisting of a supercritical CO_2 extracted seed oil (EPSO) product. Because a major constituent of pomegranate seeds is believed to be punicic acid, this material was purchased by MDACC for comparison against the Rimonest products.

Analytical methodology
Punicic acid as well as PPSE and EPSO products were analyzed by LC/MS/MS (tandem mass spectrometry using an electrospray interface set in a negative ion mode).

HPLC Column:	Waters Xterra 3.5 um C18 (2 x 30 mm)
Mobile phase A:	10 mM ammonium acetate, pH 8.5
Mobile phase B:	Methanol

The PPSE samples were diluted in methylene chloride to a concentration of 10 mg/ml. All subsequent dilutions were made in 70:30 methanol: 10 mM ammonium acetate, pH 8.5.

Punicic acid was readily detected using a selected ion channel set at 277.1. Concentrations down to 0.1 ng/ml were readily detected. Concentrations from 0.1 to 1000 ng/ml produced an excellent linear response (0.9999) in the LC/MS/MS. Various dilutions of the PPSE and EPSO products were compared to the punicic acid standard curve.

Product	Relative Punicic acid content
PPSE	1.19%
EPSO	2.10%

Comment on analysis:
The analyses of the pomegranate seed oil products were undertaken with the assumption that punicic acid would be a major component. Information made available to us suggested that a content of up to 80% punicic acid could be expected in pomegranate seed oil. Therefore the punicic acid content of less than 3% was a surprise and begs the question as to what compounds/oils are actually in the PPSE and EPSO products.

Cytotoxicity
The relative ability of punicic acid and the pomegranate seed oil products to inhibit the growth of a small series of human malignant cancer cell lines was evaluated using an "MTT" assay. This assay measures the relative inhibition of cell growth over a defined period of time (i.e. 72 hr) after continuous exposure of cells to drug. Relative cell growth of treated cells is compared to that of nontreated cells.

Punicic acid was initially dissolved in dimethyl sulfoxide and subsequently diluted in cell culture media without serum.

Cell line	Punicic acid (ug/ml)	338: PPSE (ug/ml)	339: EPSO (ug/ml)
A549 (lung)	31.4	>100	>100
HT 29 (colon)	7.4	>100	>100
K562 (leukemia)	4.1	>100	>100
PC3 (prostate)	20.4	>100	>100

Data are reported as relative IC50 values (that concentration inhibiting the growth of cell lines by 50% after 72 hours of continuous exposure to the test product). The "concentration" of EPSO and PPSE were based on the relative weight of products and not on punicic acid content.

These tests are being repeated using higher concentrations of PPSE and EPSO. Obviously, the products had no cytotoxicity at concentrations up to and including 100 ug/ml.

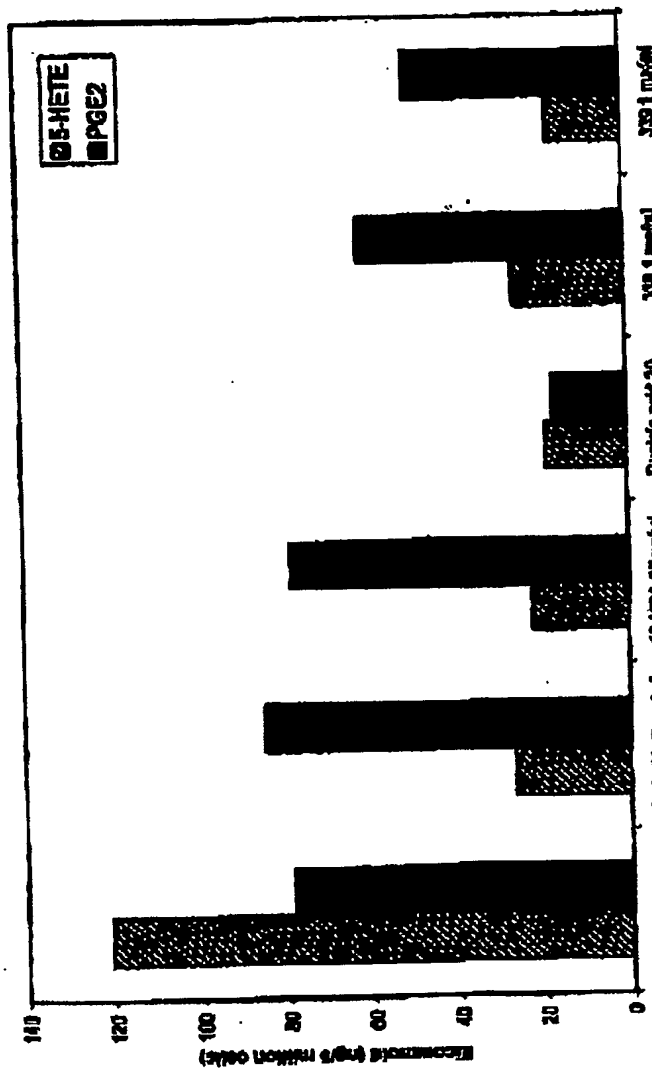
Effects on eicosanoid metabolism

We have recently developed a novel LC/MS/MS based assay to look at the effect of drugs on multiple eicosanoid products within cells in culture (*Analytical Biochemistry* 297: 183-190, 2001). The RBL-1 cell line was exposed to two different branched chain fatty acids (i.e. 12-methyltetradecanoic acid and 13-methylpentadecanoic acid) of interest to us as well as punicic acid or the PPSE or EPSO products. As seen in Figure 1, all products tested resulted in a selective inhibition of 5-lipoxygenase activity denoted as a decreased synthesis of 5-HETE. Of particular interest was the coordinate decrease in cyclooxygenase activity achieved by incubation of cells with punicic acid. We have not previously seen this with the "bioactive" lipids currently under study in our lab. While this study has been repeated with similar results, we have not done the statistics to compare relative inhibition of COX and LOX activity for each product. However, it appears that EPSO product also has an ability to inhibit COX as well as LOX activity. It is of interest therefore that the EPSO had a punicic acid content that was twice that of the PPSE product.

Comments:

- As previously mentioned, the low content of punicic acid in the PPSE and EPSO products was unexpected.
- The relative inhibition of both 5-LOX and COX activity of punicic acid was of great interest to us as we have not previously seen this pharmacologic activity in a bioactive lipid.
- Although the pomogranate seed oil contains many pharmacophores of interest, the question arises as to whether the relative seed oil content of punicic acid might be manipulated. That is, it could be imagined that a seed oil extract high in punicic acid content might offer certain advantages over one relatively low in punicic acid content.
- Although we believe our analyses of punicic acid content in the seed oil products to be accurate, we encourage the company to seek analyses elsewhere. It is strongly suggested that a laboratory specifically doing lipid analyses (perhaps by GC/MS) may be appropriate.

The effect of various agents on 5-HETE and PGE2 formation in RBL-1 cells



RBL-1 cells (5×10^6) were preincubated with the reagents at indicated concentrations at 37 C for 5 min. Then an aliquot of 2.5 ul of calcium ionophore A23187 (1 mM) was added followed with addition of arachidonic acid (100 uM). The eicosanoids were extracted and analyzed by LC/MS/MS. All the reagents were dissolved in DMSO.

Angiogenesis 6: 000–000, 2003.

Preliminary studies on the anti-angiogenic potential of pomegranate fractions *in vitro* and *in vivo*

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Abstract

We previously showed pomegranate seed oil and fermented juice polyphenols to retard oxidation and prostaglandin synthesis, to inhibit breast cancer cell proliferation and invasion, and to promote breast cancer cell apoptosis. Here we evaluated the anti-angiogenic potential of these materials in several ways. We checked a possible effect on angiogenic regulation by measuring vascular endothelial growth factor (VEGF), interleukin-4 (IL-4) and migration inhibitory factor (MIF) in the conditioned media of estrogen sensitive (MCF-7) or estrogen resistant (MDA-MB-231) human breast cancer cells, or immortalized normal human breast epithelial cells (MCF-10A), grown in the presence or absence of pomegranate seed oil (SESCO) or fermented juice polyphenols (W). VEGF was strongly downregulated in MCF-10A and MCF-7, and MIF upregulated in MDA-MB-231, overall showing significant potential for downregulation of angiogenesis by pomegranate fractions. An anti-proliferative effect on angiogenic cells was shown in human umbilical vein endothelial cell (HUVEC) and in myometrial and amniotic fluid fibroblasts, and inhibition of HUVEC tubule formation demonstrated in an *in vitro* model employing glass carrier beads. Finally, we showed a significant decrease in new blood vessel formation using the chicken chorioallantoic membrane (CAM) model *in vivo*. 'In sum, these varied studies employing different models in different laboratories overall demonstrate for the first time an anti-angiogenic potential of pomegranate fractions, suggesting further *in vivo* and clinical investigations'.

Abbreviations: ■.

Introduction

Angiogenesis is a critical process for the development and progression of cancer [1], and an important pharmacological target both for cancer prevention [2] and treatment [3]. In screening potential drug candidates against angiogenesis, a broad range of both *in vitro* and *in vivo* assessments provides the highest level of certainty for the potential drug's efficacy [4]. In this context, the present study was undertaken to test pomegranate fractions in multiple pharmacological settings pertinent to angiogenesis. Specifically, we looked at fibrocyte proliferation, production of vascular regulatory molecules by normal and cancerous breast cells, tubule formation and *in vivo* blood vessel formation in the chick embryo chorioallantoic membrane (CAM). Pomegranate (*Punica granatum*) is an ancient fruit of great medical interest. We previously showed extracts to exert suppressive and chemopreventive effects against breast cancer *in vitro* and in an animal organ culture model [5]. Pomegranate juice and peels are rich in estrogenic flavonoids like luteolin, which, elsewhere, have been shown to be anti-angiogenic [6, 7] or to inhibit factors which promote angiogenesis, e.g., bFGF (basic FGF) [8]. Furthermore, pomegranate seed oil is largely composed of a three-double-bond, conjugated linoleic acid

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(CLA), punicic acid [9]. The more common two-double-bond types of CLA found in bovine flesh and milk inhibits angiogenesis [10]. These grounds suggested that pomegranate might also possess anti-angiogenic activities.

Fibrocytes are important in angiogenesis because they lay its requisite intercellular infrastructure [11]. They also secrete extracellular matrix degrading enzymes (e.g., matrix metalloproteinase 9), which promote endothelial cell invasion, and secrete pro-angiogenic factors such as vascular endothelial growth factor (VEGF), basic fibrocyte growth factor (bFGF) and the interleukins that, favor endothelial cell proliferation and/or tube cell formation, but very few anti-angiogenic factors [12]. For these reasons, we first examined the effect of the pomegranate fractions on fibrocyte proliferation. The fibrocytes studied were obtained under informed consent during caesarean sections from the pregnant myometrium and amniotic fluid. Angiogenesis is important during the rapid growth of the myometrium and amniotic membranes during pregnancy, which provide a laboratory-like setting for studying angiogenesis independent of the tumor environment [13].

Next, we turned our attention to some of those specific tumor-derived factors, that regulate angiogenesis. These included the aforementioned VEGF, interleukin 4 (IL-4) and migration inhibitory factor (MIF).

Angiogenesis is favored by upregulation of VEGF [14], IL-4 [15] and MIF [16]. We measured these factors in the conditioned media of normal and cancerous human breast cells grown in the presence and absence of the pomegranate fractions.

We also studied the direct effects of pomegranate fractions on endothelial cells, as these provide the lining for the new blood vessels themselves. We examined proliferation of human umbilical vein endothelial cells (HUVEC), and on tubule formation by HUVEC, an event recognized as necessary for angiogenesis [17].

Though *in vitro* tests provide the best opportunity to examine mechanisms, *in vivo* testing is also essential to study the angiogenic effect in the complex organismic environment. To address this requirement, we examined the effect of the pomegranate extracts also on new blood vessel formation in the chick embryo chorioallantoic membrane (CAM).

Materials and methods

Pomegranate extracts

Pomegranate extracts deriving from the 2000 crop of pomegranates of the 'Wonderful' cultivar, bio-organically grown on Kibbutz Sde Eliahu, Israel, were employed. These included pomegranate fermented juice (pomegranate 'wine') polyphenols (W), pomegranate pericarp polyphenols (P), cold-pressed pomegranate seed oil (SEPO), supercritical fluid extracted pomegranate seed oil (SESCO), pomegranate seed oil polyphenols (O), and unsaponified pomegranate seed oil fraction (N) all provided by Rimonest Ltd. (Haifa, Israel).

Measurement of fibroblast proliferation using continuous and pulse-labeling

Human fibroblasts were derived from amniotic membrane and from pregnant myometrium from obstetrical patients, at the time of elective Caesarian section, who gave informed consent for the disposition of their surgically removed tissues. Approval from the Gifu University Institutional Review Board (IRB) was also obtained beforehand. The fibroblasts were seeded at confluence in plastic 24-well plates at 100,000 cell/cm^2. Monolayer cultures were preincubated in modified minimum essential medium (MEM) with 1% FBS for 2 days and were subjected to concurrent exposures of different pomegranate fractions (W, P, O, SEPO) at 37 °C. ^{3}H-thymidine uptake was examined by continuous labeling for 24 h or by using 2-h pulse labeling at 24 h with 1 μCi/well, and uptake was quenched by aspiration of the medium. The cells were washed with ice-cold 0.5% trichloroacetic acid, and the radioactivity was counted.

Growth of cells and measurement of VEGF, IL-4 and MIF in conditioned media

Three different human breast cell lines, estrogen-sensitive MCF-7 cancer cells, estrogen-resistant MDA-MB-231 cancer cells, and immortalized normal MCF-10A epithelial cells, were grown in culture at 37 °C for 24 h in humidified 5% CO_2 in air in the presence of the Rimonest individual pomegranate fractions W, P, SEPO and SESCO. After 72 h of growth, cultures were centrifuged to obtain the supernatants (conditioned media), and these were immediately packed on dry ice and subsequently analyzed for VEGF, MIF and IL-4 with the commercial R&D Elisa kits (R&D systems, Minneapolis, Minnesota) as previously described [18].

Growth of HUVEC and measurement of proliferation using the MTT assay

HUVECs were obtained from Cell Systems (Kirkland, Washington) and cultured in complete medium (MCDB-131 containing 10% fetal bovine serum (FBS; Moregate, Melbourne, Australia) containing 10 μg/ml endothelial cell growth supplement (Upstate, Lake Placid, New York), 10 ng/ml epidermal growth factor (Sigma, St. Louis, Missouri) and 10 μg/ml heparin (Sigma, St. Louis, Missouri).

HUVEC proliferation was determined using a 3-(4,5-dimethylthaizol-2-yl)-2,5-diphenyltetrasodium bromide (MTT) assay according to the manufacture's instructions (Promega, Tokyo, Japan). HUVECs (1 × 103 cell per 0.1 ml of growth medium) were dispensed within gelatin-coated 96-well culture plates (Iwaki, Tokyo, Japan). The cells were incubated at 37 °C for 4 h in a CO_2 incubator,

and the medium was changed to that containing various concentrations of a test sample. After 72 h incubation, 100 μl of MTT reagent was added to each well. Following additional 4-h incubation at 37 °C, 10 μl of solubilization solution was added to dissolve the formazan crystals, and the absorbance was then measured at 570 nm using a MTP-120 microplate reader (Corona Co. Ltd., Ibaraki, Japan). Wells without cells were used as blanks and were subtracted as background from each sample. Results were expressed as percentage of control.

Assay for plasminogen activator activity

Plasminogen activator activity from HUVECs was determined as described previously [19]. In brief, HUVECs (9 × 105 cells/dish were cultured in gelatin-coated 35-mm dishes containing 2 ml of complete medium at 37 °C for 24 h in a humidified chamber under 5% CO_2, and then incubated in serum-free growth medium containing 0.1% bovine serum albumin in the presence or absence of a test sample (or medroxyprogesterone acetate) for 18 h. The medium was aspirated, and then the cells attached to dishes were washed twice with phosphate-buffered saline and then extracted with 0.5 ml of 0.5% Triton X-100 in phosphate-buffered saline. Plasminogen activator activity in the cell extracts was determined. Plasminogen activator activity was determined at 37 °C and pH 7.4 in 0.1 M Tris–HCl containing human plasminogen (Roche, Tokyo, Japan) and H-D-Val-Leu-Lys-p-nitroanilide (S-2251; Chromogenix, Molndal, Sweden). Protein concentrations were determined with bovine serum albumin as a standard according to the manufacturer's instructions (DC protein assay; Bio-Rad, Hercules, California).

Measurement of tubule formation in HUVEC

Stable complexes consisting of HUVEC adherent to microcarrier beads were established according to a previously described method, and tubule formation among the HUVEC assessed by visually counting the number of tubules extending from each bead [20]. The complexes were used in 12-well plates, and tubule formation stimulated with conditioned media from estrogen-resistant MDA-MB-435 breast cancer cells (grown in MEM, 5% FBS, 1% antibiotic-antimycotic solution, 1% sodium pyruvate, 2% MEM vitamin solution, non-essential amino acid solution, pH adjusted to 7.4), VEGF, and bFGF, all from Sigma, St. Louis, Missouri). The pomegranate fractions were added to the experimental wells following stimulation with the conditioned media, and tubule formation quantified daily by visual inspection at 10×.

Chick embryo CAM assay

The CAM assay was established as previously described [21–23]. Briefly, the CAMs of 5-day-old chick embryos were treated with ethylene–vinyl acetate copolymer 40 pellets containing, or not containing, pomegranate extracts W or P at 37 °C for 2 days in a humidified egg incubator, after which an appropriate volume of a 20% fat emulsion was injected into the choriolantois to show the vascular network better. The anti-angiogenic response was assessed as positive when the avascular zone exceeded 3 mm in diameter; only the frequency was monitored. This experiment was approved by the Committee on the Ethics of Animal Experiments of the Tokyo Metropolitan Institute of Medical Science, and was carried out in accordance with the Guidelines for Animal Experiments of The Tokyo Metropolitan Institute of Medical Science.

Results

Suppression of fibroblast proliferation by pomegranate fractions

Figure 1 demonstrates the effect of pomegranate fractions W and P on proliferation of myometrial and amniotic fluid fibroblasts using continuous labeling. Pulse labeling generated a similar pattern (not shown). Pomegranate seed oil polyphenols (O) and cold pressed pomegranate seed oil (SEPO) exerted more modest inhibitory effects (not shown).

VEGF, IL-4 and MIF in conditioned media

Pomegranate fermented juice polyphenols (W) and pomegranate seed oil (SESCO) caused downregulation of VEGF in MCF-7 estrogen-dependent breast cancer cells (Figure 2). A less pronounced downregulation trend was noted in MDA-MB-231 estrogen-resistant breast cancer cells (Figure 3). The most pronounced and sustained downregulation was seen in the MCF-10A immortalized normal breast epithelial cells (Figure 4). Pomegranate pericarp polyphenols (P) effected a downregulation similar, but milder, to that seen for the fermented juice polyphenols (W) in all cases (data not shown). No difference in IL-4 expression was noted

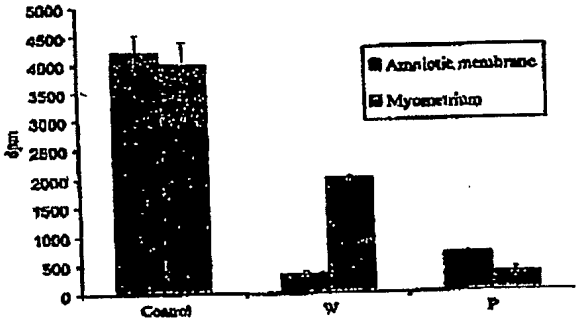

Figure 1. Effects of pomegranate fractions W and P (35 μg/ml) on proliferation of human myometrial and amniotic fluid fibrocytes. Samples obtained under informed consent.

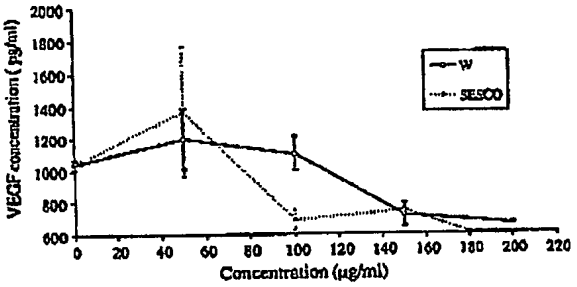

Figure 2. Effects of pomegranate seed oil (SESCO) and pomegranate fermented juice polyphenols (W) on VEGF expression by MCF-7 estrogen-sensitive human breast cancer cells.

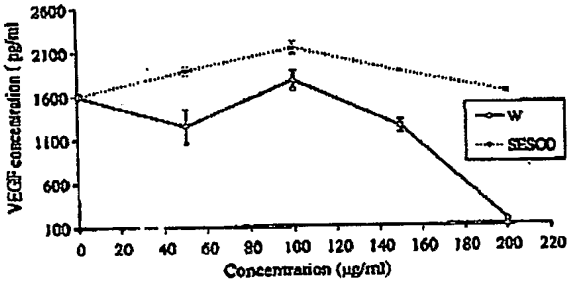

Figure 3. Effects of pomegranate seed oil (SESCO) and pomegranate fermented juice polyphenols (W) on VEGF expression by MDA-MB-231 estrogen-resistant human breast cancer cells.

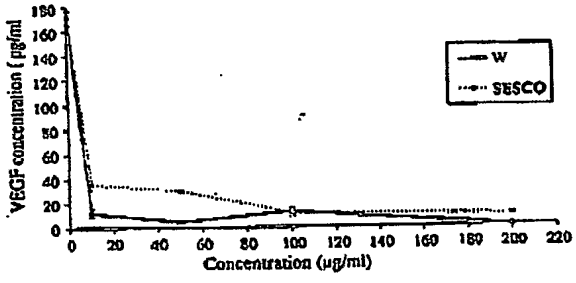

Figure 4. Effects of pomegranate seed oil (SESCO) and pomegranate fermented juice polyphenols (W) on VEGF expression by MCF-10 immortalized normal human breast epithelial cells.

from controls in all cases. A dose-dependent upregulation of MIF was caused in MDA-MB-231 cells by both W and SESCO (Figure 5), and a brief upregulation by both fractions noted in MCF-10A at low dose only (Figure 6). The expression of MIF was not significantly affected by either fraction in MCF-7.

Proliferation of HUVEC

Figure 7 reveals a modest but significant inhibitory effect of W on HUVEC at even very low concentrations.

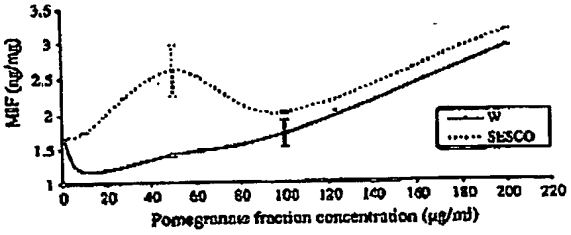

Figure 5. Effects of pomegranate fermented juice polyphenols (W) and pomegranate seed oil (SESCO) on MIF expression in MB-MDA-231 estrogen-receptor-negative human breast cancer cells.

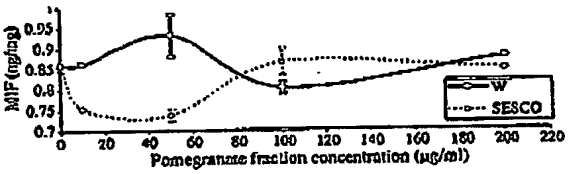

Figure 6. Effect of pomegranate fermented juice polyphenols (W) and pomegranate seed oil (SESCO) on MIF expression in MCF-10A immortalized normal human breast epithelial cells.

Plasminogen activator activity

Concentrations of W ranging from 10^{-6} to 10 µg/ml had no significant effect on plasminogen activator activity (data not shown).

Tubule formation by HUVEC

Figure 8 shows the effect of 10 µg/ml of different pomegranate fractions, or combinations, on suppression of tubule formation from HUVEC. Note there is a suggestion of an additive effect from the combination of W and O. A similar pattern was noted at 5 µg/ml (not shown).

Suppression of vascularization in chick embryo CAM

Figure 9A shows the suppression of vascularization in the CAM model by W, while P was without effect. Figure 9B demonstrates the same result graphically

A simplified summary of the effects of all pomegranate extracts used in this study in all experimental settings is given in Table 1.

Discussion

Overall, this study helps to establish the potential of pomegranate fractions as potential adjuncts to both the chemoprevention and chemotherapy of breast cancer. The particularly striking suppression of VEGF in the most highly differentiated cell line, MCF-10A, is particularly suggestive of a role of these extracts in

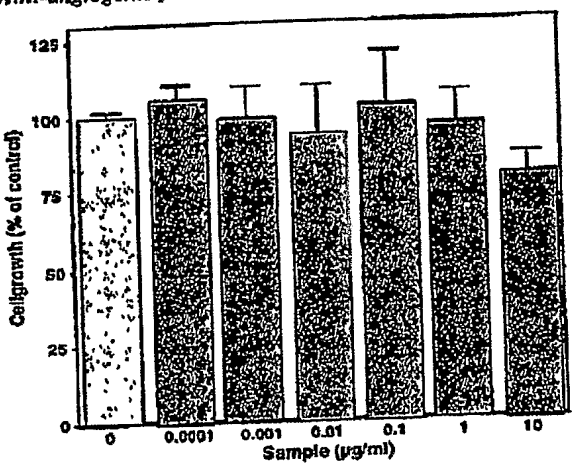

Figure 7. Effects of pomegranate fractions W and P on proliferation of HUVEC as measured by the MTT assay.

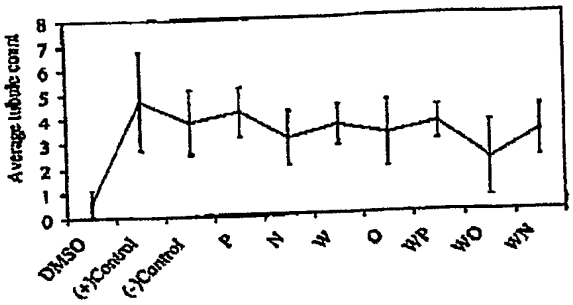

Figure 8. Effects of 10 µg/ml of selected pomegranate fractions and combinations on tubule formation in HUVEC that was stimulated by conditioned medium and soluble factors.

chemoprevention. That IL-4 was not affected in any cell line, helps to highlight the specificity of the effect of the extracts on VEGF.

Upregulation of MIF specifically in MDA-MB-231 is interesting in light of the seeming insensitivity of VEGF expression to the pomegranate fractions in this line. Thus, pomegranate fractions may possess anti-angiogenic effects which involve downregulation of an angiogenic promoter (VEGF) in some types of cancer cells (MCF-7), and upregulation of angiogenic suppressors (MIF) in other cell types (MDA-MB-231). Such multiple cytokine or chemokine targeting by the fractions is a desirable attribute for anticancer therapy [24].

Oxidative stress is an important trigger for angiogenesis [25, 26], and the mechanism for this trigger likely

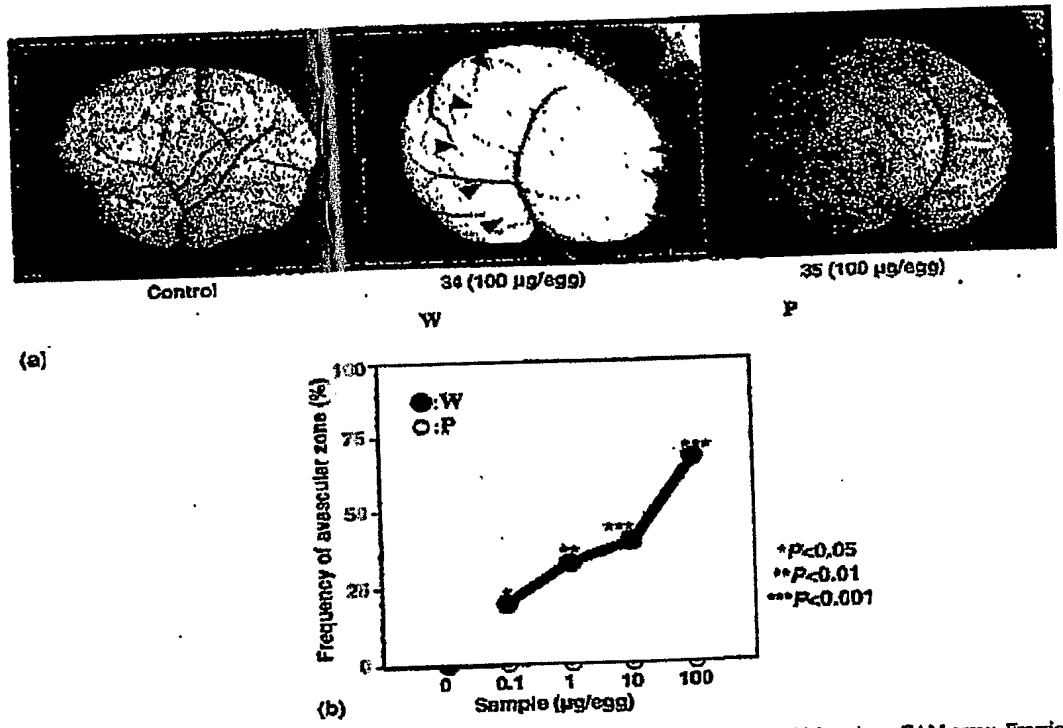

Figure 9. (A) Photomicrograph showing effect of pomegranate fraction W on neovascularization in chick embryo CAM assay. Fraction P was without effect. (B) Effect of pomegranate fraction W on neovascularization in chick embryo CAM. Fraction P was without effect.

Table 1. Summary of effects of pomegranate fractions on pharmacological parameters pertinent to angiogenesis.

Fraction	Test					VEGF downreg.			MIF upreg.			IL-4 upreg.			CAM
	Myometrial fibrocyte proliferation inhibition	Amniotic fibrocyte proliferation inhibition	HUVEC proliferation inhibition	HUVEC tubule formation inhibition	MCF-7	MB-MDA-231	MCF-10-A	MCF-7	MB-MDA-231	MCF-10-A	MCF-7	MB-MDA-231	MCF-10-A	angiogenesis inhibition *in vivo*
P	2	2	–	–	1	0	3	0	0	0	0	0	0	0
W	3	2	2	2	1	0	3	0	1	1	0	0	0	3
O	1	2	–	1	–	–	–	–	–	–	–	–	–	–
N	–	–	–	0	–	–	–	–	–	–	–	–	–	–
BOT	–	–	–	–	1	0	3	0	0	0	0	0	0	–
SEPO	1	2	–	–	1	0	2	0	0	0	0	0	0	–
SESCO	–	–	–	–	2	0	3	0	2	0	0	0	0	–
WP	–	–	–	1	–	–	–	–	–	–	–	–	–	–
WO	–	–	–	2	–	–	–	–	–	–	–	–	–	–
WN	–	–	–	1	–	–	–	–	–	–	–	–	–	–

P = pomegranate pericarp polyphenols; W = pomegranate fermented juice polyphenols; O = pomegranate seed oil polyphenols; N = non-saponified fraction of pomegranate seed oil; BOT = botanical fraction of pomegranate seed cake extract; SEPO = cold pressed pomegranate seed oil; SESCO = supercritical fluid extracted pomegranate seed oil; WP = 1:1 mixture of pomegranate fermented juice and pericarp polyphenols; WO = 1:1 mixture of pomegranate fermented juice and seed oil polyphenols; WN = 1:1 mixture of pomegranate fermented juice polyphenols and non-saponified fraction of pomegranate seed oil; HUVEC = human umbilical vein endothelial cells; VEGF = vascular endothelial growth factor; MIF = migration inhibitory factor; IL-4 = interleukin 4; CAM = chicken chorioallantoic membrane; MCF-7 = an estrogen receptor positive human breast cancer cell line; MB-MDA-231 = an estrogen receptor negative human breast cancer cell line; MCF-10A = an immortalized normal human breast epithelial cell line; downreg. = downregulation; upreg. = upregulation.

Scale: (3) = strong effect; (2) = moderate effect; (1) = mild effect; (0) = no significant effect; (–) = not tested.

involves NFkappaB [27]. Significantly, NFkappaB is important in mediating the antioxidant activity of pomegranate fermented juice polyphenols [28]. Therefore, the downregulation of VEGF and upregulation of MIF might possibly involve anti-oxidative mechanisms.

Pomegranate fractions, especially the seed oil, are known to possess estrogenic activity [29]. Estrogens act on vascular endothelium [30] both through estrogen receptors [31] and *via* NFkappaB [32]. Thus, the effects here observed might also involve known patterns of estrogenic influence [33]. Specifically, the relative sensitivity of the estrogen receptor positive MCF-7 to downregulation of VEGF by the pomegranate fractions over the estrogen receptor negative MDA-MB-231 may partially be related to these mechanisms [34].

In addition to these arguments, pomegranate seed oil, as previously stated, consists largely of conjugated fatty acids similar to the CLA in bovine products. Recently, CLA has been shown to exert its anti-angiogenic activity possibly through blockade of another angiogenic promotor found in fibroblasts, i.e., bFGF [34]. The punicic acid in pomegranate seed oil might act likewise.

Furthermore, punicic acid inhibits prostaglandin formation [35]. Inhibition of prostaglandin biosynthesis by inhibiting cyclooxygenase also downregulates VEGF expression [36], and upregulates MIF expression [37]. In that the same pattern of VEGF downregulation and MIF upregulation was observed herein from pomegranate fractions suggests inhibition of cyclooxygenase and/or other eicosanoid enzymes as a likely mechanism.

Generally, the present study highlights anti-angiogenic potential for pomegranate extracts. This has been demonstrated herein from suppression of fibroblast growth, suppression of HUVEC growth and also HUVEC tubule formations. Angiogenic promoter molecules have been suppressed, and angiogenic inhibitory molecules promoted. Finally, inhibition of angiogenesis has been demonstrated *in vivo*. Undoubtedly, the results from the different pomegranate fractions, with their different chemistries, rely upon different and multiple mechanisms.

Such pharmacological redundancy is a valuable and an important attribute of the pomegranate that may reinforce its applicability as an inhibitor of angiogenesis, and as an anti-cancer agent. The present study is in no way meant to be seen as comprehensive, as it leaves many gaps. Most notably, not all the fractions were tested in all of the models. However, in sum, there are sufficient indications to indicate anti-angiogenic potential of these extracts. In other settings [38] we proved that the different fractions (e.g., P, W and SEPO or SESCO) acted synergistically to suppress prostate cancer proliferation and invasion, and also to suppress the activity of phospholipase A2. Conceivably, such kind of synergistic interaction may also apply to the activities of these extracts against angiogenesis.

In fact, the extracts employed in the present study are seen as components of a complex pomegranate drug in development for the prevention and/or treatment of breast and/or prostate cancer. The present preliminary studies lend credence to a more systematic examination of this agent in the context of angiogenesis.

Acknowledgements

The authors wish to thank the family of the late Jeffrey Marks for fermentation of the pomegranate juice, to Eli Merom for generosities in supplying the organically grown pomegranates, to Talia Livney for preparation of samples, and to Alexander Botvinnik for technical assistance in the preparation of the manuscript.

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ORIGINAL PAPER

Preliminary studies on the anti-angiogenic potential of pomegranate fractions *in vitro* and *in vivo*

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KEYWORDS: angiogenesis, breast cancer, chemoprevention, *Punica granatum*, VEGF, MIF

Abstract

We previously showed pomegranate seed oil and fermented juice polyphenols to retard oxidation and prostaglandin synthesis, to inhibit breast cancer cell proliferation and invasion, and to promote breast cancer cell apoptosis. Here we evaluated the anti-angiogenic potential of these materials in several ways. We checked a possible effect on angiogenic regulation by measuring Vascular Endothelial Growth Factor (VEGF), Interleukin-4 (IL-4) and Migration Inhibitory Factor (MIF) in the conditioned media of estrogen sensitive (MCF-7) or estrogen resistant (MDA-MB-231) human breast cancer cells, or immortalized normal human breast epithelial cells (MCF-10A), grown in the presence or absence of pomegranate seed oil (SESCO) or fermented juice polyphenols (W). VEGF was strongly downregulated in MCF-10A and MCF-7, and MIF was upregulated in MDA-MB-231, overall showing significant potential for downregulation of angiogenesis by pomegranate fractions. An anti-proliferative effect on angiogenic cells was shown in human umbilical vein endothelial cell (HUVEC) and in myometrial and amniotic fluid fibroblasts, and inhibition of HUVEC tubule formation demonstrated in an *in vitro* model employing glass carrier beads. Finally, we showed a significant decrease in new blood vessel formation using the chicken chorioallantoic membrane (CAM) model *in vivo*. *In sum, these varied studies employing different models in different laboratories overall demonstrate for the first time an anti-angiogenic potential of pomegranate fractions, suggesting further* in vivo *and clinical investigations.*

Introduction

Angiogenesis is a critical process for the development and progression of cancer (Eatock MM, Schatzlein A, Kaye SB [1]), and an important pharmacological target both for cancer prevention (Pfeffer U, Ferrari N, Morini M et al [2]) and treatment (Scappaticci FA [3]). In screening potential drug candidates against angiogenesis, a broad range of both *in vitro* and *in vivo* assessments provides the highest level of certainty for the potential drug's efficacy (Auerbach R, Lewis R, Shinners B [4]). In this context, the present study was undertaken to test pomegranate fractions in multiple pharmacological settings pertinent to angiogenesis. Specifically, we looked at fibroblast proliferation, production of vascular regulatory molecules by normal and cancerous breast cells, tubule formation and *in vivo* blood vessel formation in the chick embryo chorioallantoic membrane (CAM).

Pomegranate (*Punica granatum*) is an ancient fruit of great medical interest. We previously showed extracts to exert suppressive and chemopreventive effects against breast cancer *in vitro* and in an animal organ culture model (Kim ND, Mehta R, Yu W et al [5]). Pomegranate juice and peels are rich in estrogenic flavonoids like luteolin, quercetin and kaempferol (Elswijk DA, Schobel, UP, Lansky EP et al [6]), which, elsewhere, have been shown to be anti-angiogenic (Fotsis T, Pepper MS, Montesano R [7], Le Marchand L [8]) or to inhibit factors which promote angiogenesis, *e.g.*, bFGF (basic FGF) (Sartippour MR, Heber D, Zhang L [9]). Furthermore, pomegranate seed oil is largely composed of a three-double-bond, conjugated linoleic acid (CLA), punicic acid (Schubert SY, Lansky EP, Neeman I [10]). The more common two-double-bond types of CLA found in bovine flesh and milk inhibit angiogenesis (Masso-Welch PA,

Zangani D, Ip C [11]). These grounds suggested that pomegranate might also possess anti-angiogenic activities.

Fibrocytes ("Fibroblast" and "fibrocyte" are roughly synonymous, fibroblast implying a less mature cell) are important in angiogenesis because they lay its requisite intercellular infrastructure (Kunz-Schughart LA, Knuechel R. [12]). They also secrete extracellular matrix degrading enzymes (e.g., matrix metalloproteinase 9) which promote endothelial cell invasion, and secrete pro-angiogenic factors such as VEGF (vascular endothelial growth factor), bFGF (basic fibrocyte growth factor) and the interleukins that, favor endothelial cell proliferation and / or tube cell formation, but secrete very few anti-angiogenic factors (Hartlapp I, Abe R, Saeed RW et al [13]). For these reasons, we first examined the effect of the pomegranate fractions on fibrocyte proliferation. The cells studied were obtained under informed consent during caesarean sections from the pregnant myometrium and amniotic fluid. Angiogenesis is important during the rapid growth of the myometrium and amniotic membranes during pregnancy, which provide a laboratory-like setting for studying angiogenesis independent of the tumor environment (Kapiteijn K, Koolwijk P, Van Der Weiden R *[14]*).

Next, we turned our attention to some of those specific tumour-derived factors that regulate angiogenesis. These included the aforementioned VEGF, interleukin 4 (IL-4) and migration inhibitory factor (MIF). Angiogenesis is favored by upregulation of VEGF (Toi M, Matsumoto T, Bando H [15]), and downregulation of IL-4 (Nagai S, Toi M [16]) and MIF (Bando H, Matsumoto G, Bando M et al [17]). We measured these factors in the conditioned media of

normal and cancerous human breast cells grown in the presence and absence of the pomegranate fractions.

We also studied the direct effects of pomegranate fractions on endothelial cells, as these provide the lining for the new blood vessels themselves. We examined proliferation of human umbilical vein endothelial cells (HUVEC), and on tubule formation by HUVEC,, an event recognized as necessary for angiogenesis (Imamura M [18]), and plasminogen activator activity, which promotes extracellular membrane degradation (Sidenius N, Blasi F [19]).

Though *in vitro* tests provide the best opportunity to examine mechanisms, *in vivo* testing is also essential to study the angiogenic effect in the complex organismic environment. To address this requirement, we examined the effect of the pomegranate extracts also on new blood vessel formation in the chick embryo chorioallanatoic membrane (CAM).

Materials and methods

Pomegranate extracts

Pomegranate extracts deriving from the 2000 crop of pomegranates of the "Wonderful" cultivar, bio-organically grown on Kibbutz Sde Eliahu, Israel, were employed. These included pomegranate fermented juice (pomegranate "wine") polyphenols (W), pomegranate pericarp polyphenols (P), cold-pressed pomegranate seed oil (SEPO), supercritical fluid extracted pomegranate seed oil (SESCO), pomegranate seed oil polyphenols (O), and unsaponified pomegranate seed oil fraction (N), all provided by Rimonest Ltd. (Haifa, Israel).

Measurement of Fibroblast Proliferation using Continuous and Pulse-Labeling

Human fibroblasts were derived from amniotic membrane and from pregnant myometrium from obstetrical patients, at the time of elective Caesarian section, who gave informed consent for the disposition of their surgically removed tissues. Approval from the Gifu University Institutional Review Board (IRB) was also obtained beforehand. The fibroblasts were seeded at confluence in plastic 24-well plates at 100,000 cell / cm^2. Monolayer cultures were preincubated in modified minimum essential medium (MEM) with 1% FBS for 2 days and were subjected to concurrent exposures of different pomegranate fractions (*W, P, O, SEPO*) at 37 C. ^{3}H-thymidine uptake was examined by continuous labeling for 24 hours or by using 2-hour pulse labeling at 24 hours with 1 μCi/well, and uptake was quenched by aspiration of the medium. The cells were washed with ice-cold 0.5 % trichloroacetic acid, and the radioactivity was counted.

Growth of Cells and Measurement of VEGF, IL-4 and MIF in Conditioned Media

Three different human breast cell lines, estrogen-sensitive MCF-7 cancer cells, estrogen-resistant MDA-MB-231 cancer cells, and immortalized normal MCF-10A epithelial cells, were grown in culture at 37° C for 24 hours in humidified 5% CO_2 in air in the presence of the Rimonest individual pomegranate fractions *W, P, SEPO* and *SESCO*. After 72 hours of growth, cultures were centrifuged to obtain the supernatants (conditioned media), and these were immediately packed on dry ice and subsequently analyzed for VEGF, MIF and IL-4 with the commercial R & D ELISA kits (R&D systems, Minneapolis, MN, USA) as previously described (Ueno T, Toi M, Saji H et al [20]).

Growth of HUVEC and Measurement of Proliferation using the MTT assay

Human umbilical vein endothelial cells (HUVECs) were obtained from Cell Systems, Kirkland, WA, USA and cultured in complete medium (MCDB-131 containing 10% fetal bovine serum (FBS; Moregate, Melbourne, Australia), and also containing 10 µg/ml endothelial cell growth supplement (Upstate, Lake Placid, NY), 10 ng/ml epidermal growth factor (Sigma, St, Louis, MO) and 10 µg/ml heparin (Sigma, St, Louis, MO).

HUVEC proliferation was determined using a 3-(4,5-dimethylthaizol-2-yl)-2,5-diphenyltetrasodium bromide (MTT) assay according to the manufacturer's instructions (Promega, Tokyo Japan). HUVECs (1 X 103 cell per 0.1 ml of growth medium) were dispensed within gelatin-coated 96-well culture plates (Iwaki, Tokyo, Japan). The cells were incubated at 37°C for 4 h in a CO2 incubator, and the medium was changed to that containing various concentrations of a test sample. After 72 h incubation, 100 µl of MTT reagent was added to each well. Following additional 4 h incubation at 37°C, 10 µl of solubilization solution was added to dissolve the formazan crystals, and the absorbance was then measured at 570 nm using a MTP-120 microplate reader (Corona Co. Ltd., Ibaraki, Japan). Wells without cells were used as blanks and were subtracted as background from each sample. Results were expressed as percentage of control.

Assay for plasminogen activator activity

Plasminogen activator activity from HUVECs was determined as described previously (Oikawa, T., Sasaki, T., Nakamura, M. et al [21]). In brief, HUVECs (9 X105 cells/dish were cultured in gelatin-coated 35-mm dishes containing 2 ml of complete medium at 37°C for 24 h in a humidified chamber under 5% CO2 , and then incubated in serum-free growth medium containing 0.1% bovine serum albumin in the presence or absence of a test sample (or medroxyprogesterone acetate) for 18 h. The medium was aspirated, and then the cells attached to dishes were washed twice with phosphate-buffered saline and then extracted with 0.5 ml of 0.5% Triton X-100 in phosphate-buffered saline. Plasminogen activator activity in the cell extracts was determined at 37°C and pH 7.4 in 0.1 M Tris-HCl containing human plasminogen (Roche, Tokyo, Japan) and H-D-Val-Leu-Lys-p-nitroanilide (S-2251; Chromogenix, Molndal, Sweden). Protein concentrations were assessed with bovine serum albumin as a standard according to the manufacturer's instructions (DC protein assay; Bio-Rad, Hercules, CA, USA).

Measurement of Tubule Formation in HUVEC

Stable complexes consisting of HUVEC adherent to microcarrier beads were established according to a previously described method, and tubule formation among the HUVEC assessed by visually counting the number of tubules extending from each bead (Fife RS, Sledge GW Jr, Sissons S et al [22]. The complexes were used in 12 well plates, and tubule formation stimulated with conditioned media from estrogen-resistant MDA-MB-435 breast cancer cells (grown in MEM, 5% FBS, 1% antibiotic-antimycotic solution, 1% sodium pyruvate, 2% MEM vitamin solution, non- essential amino acid solution, pH adjusted to 7.4), VEGF, and bFGF, all reagents from Sigma, St.Louis, MO, USA). The pomegranate fractions were added to the experimental

wells following stimulation with the conditioned media, and tubule formation quantified daily by visual inspection at 10X.

Chick embryo chorioallanatoic membrane (CAM) assay

The CAM assay was established as previously described (Oikawa T, Hirotani K, Nakamura O et al [23];, Oikawa T, Hirotani K, Ogasawara H et al [24]; Nakamura M, Katsuki Y, Shibutani Y et al [25]. Briefly, the chorioallantoic membranes of 5-day-old chick embryos were treated with ethylene –vinyl acetate copolymer 40 pellets containing, or not containing, pomegranate extracts W or P at 37° C for 2 days in a humidified egg incubator, after which an appropriate volume of a 20% fat emulsion was injected into the choriolantois to better show the vascular network. The anti-angiogenic response was assessed as positive when the avascular zone exceeded 3 mm in diameter; only the frequency was monitored. This experiment was approved by the Committee on the Ethics of Animal Experiments of the Tokyo Metropolitan Institute of Medical Science, and was carried out in accordance with the Guidelines for Animal Experiments of The Tokyo Metropolitan Institute of Medical Science.

Results

Suppression of Fibroblast Proliferation by Pomegranate Fractions

Figure 1 demonstrates the effect of pomegranate fractions W and P on proliferation of myometrial and amniotic fluid fibroblasts using continuous labeling. Pulse labeling generated a

similar pattern (not shown). Pomegranate seed oil polyphenols (O) and cold pressed pomegranate seed oil (SEPO) exerted more modest inhibitory effects (not shown).

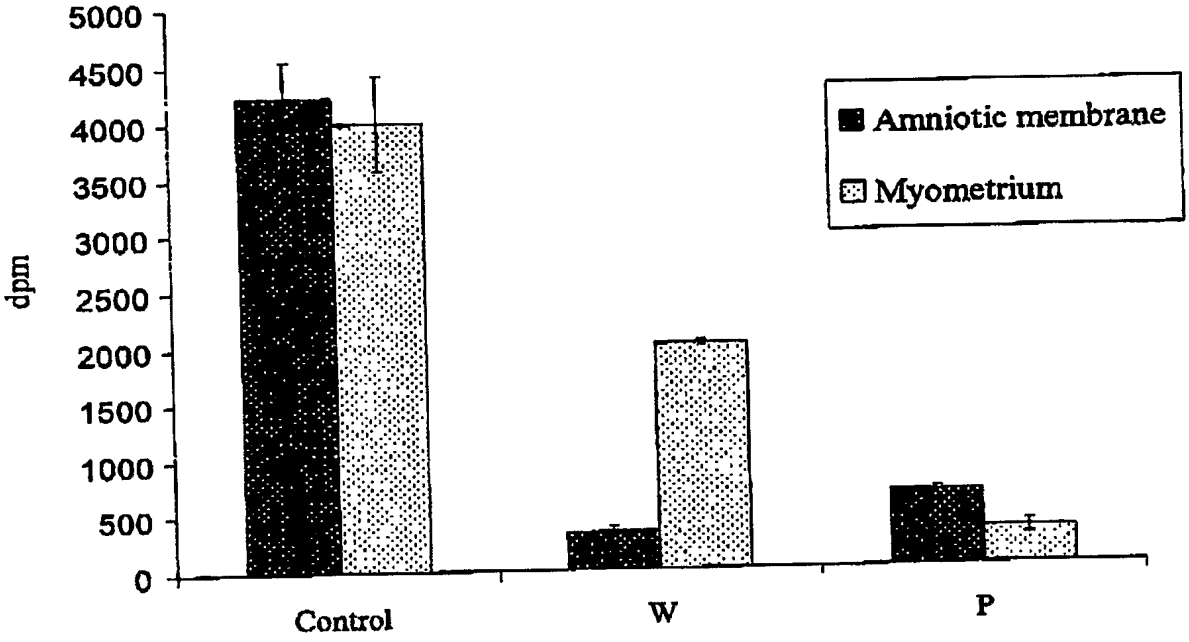


Figure 1. Effects of pomegranate fractions W and P (35 micrograms per ml) on proliferation of human myometrial and amniotic fluid fibrocytes. Samples obtained under informed consent.

Measurement of VEGF, IL-4 and MIF in conditioned media

Pomegranate fermented juice polyphenols (W) and pomegranate seed oil (SESCO) caused downregulation of VEGF in MCF-7 estrogen-dependent breast cancer cells (Figure 2). A less pronounced downregulation trend was noted in MDA-MB-231 estrogen-resistant breast cancer cells (Figure 3). The most pronounced and sustained downregulation was seen in the MCF-10A immortalized normal breast epithelial cells (Figure 4). Pomegranate pericarp polyphenols (P)

effected a downregulation similar, but milder, to that seen for the fermented juice polyphenols (W) in all cases (data not shown). No difference in IL-4 expression was noted from controls in any of the cases. A dose-dependent upregulation of MIF was caused in MDA-MB-231 cells by both W and SESCO (Figure 5), and a brief upregulation by both fractions noted in MCF-10A at low dose only (Figure 6). The expression of MIF was not significantly affected by either fraction in MCF-7.

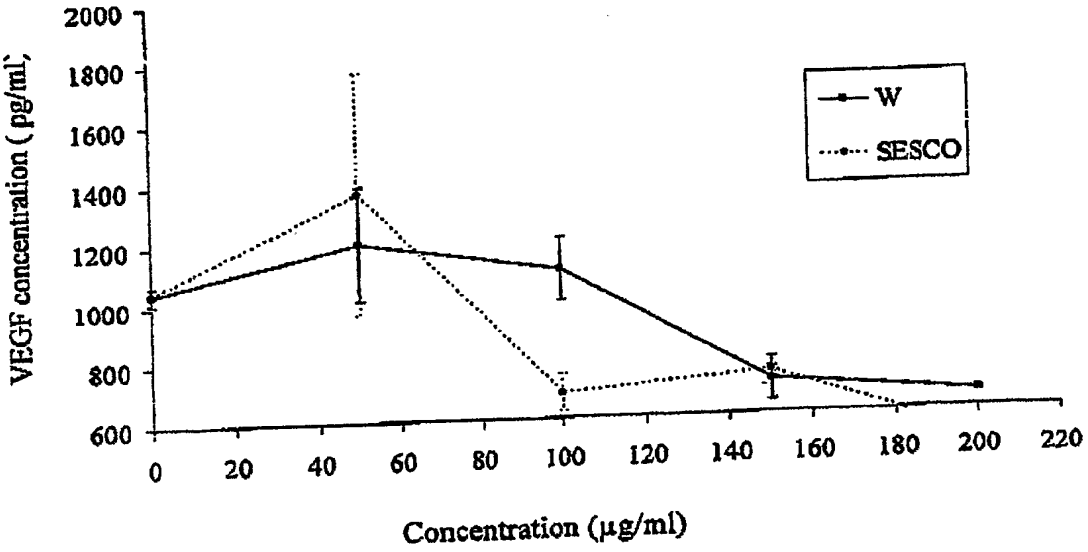


Figure 2. Effects of pomegranate seed oil (SESCO) and pomegranate fermented juice polyphenols (W) on VEGF expression by MCF-7 estrogen sensitive human breast cancer cells.

Figure 3. Effects of pomegranate seed oil (SESCO) and pomegranate fermented juice polyphenols (W) on VEGF expression by MDA-MB-231 estrogen resistant human breast cancer cells.

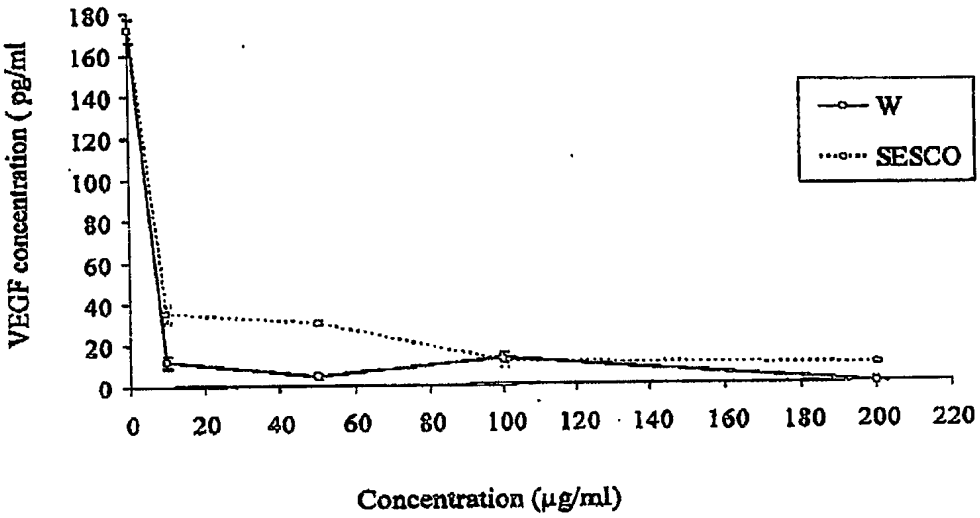


Figure 4. Effects of pomegranate seed oil (SESCO) and pomegranate fermented juice polyphenols (W) on VEGF expression by MCF-10 immortalized normal human breast epithelial cells.

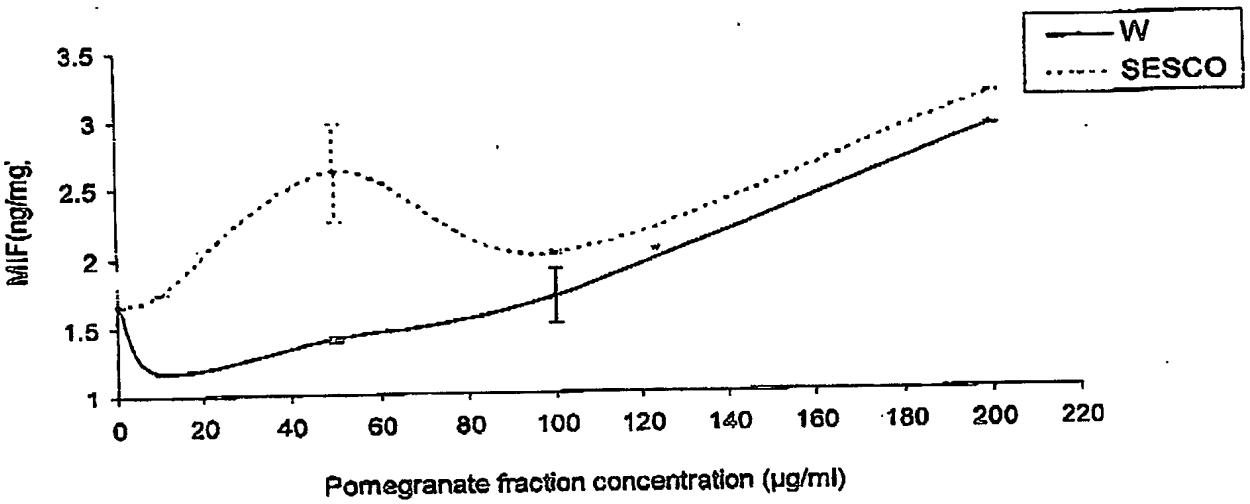


Figure 5. Effects of pomegranate fermented juice polyphenols (W) and pomegranate seed oil (SESCO) on Migration Inhibitory Factor (MIF) expression in MB-MDA-231 estrogen receptor negative human breast cancer cells.

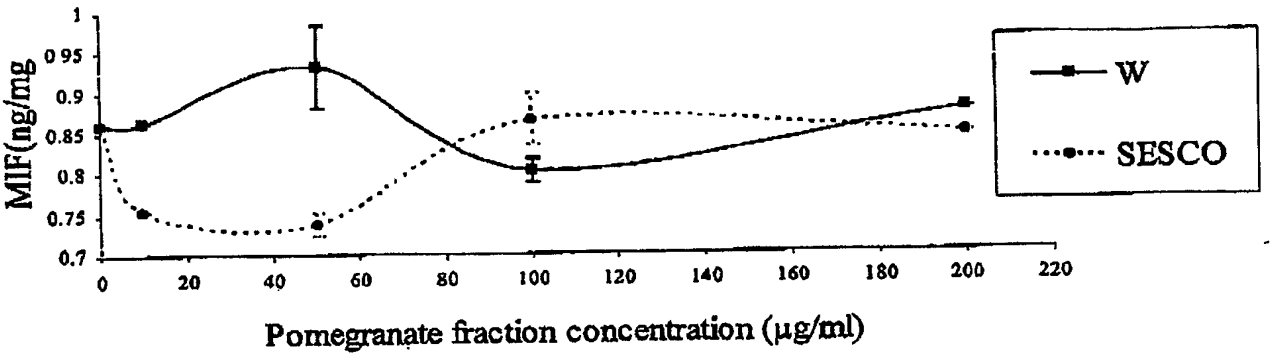


Figure 6. Effect of pomegranate fermented juice polyphenols (W) and pomegranate seed oil (SESCO) on Migration Inhibitor Factor (MIF) expression in MCF-10A immortalized normal human breast epithelial cells.

Proliferation of HUVEC

Figure 7 reveals a modest but significant inhibitory effect of W on human umbilical vein endothelial cells (HUVEC) at even very low concentrations.

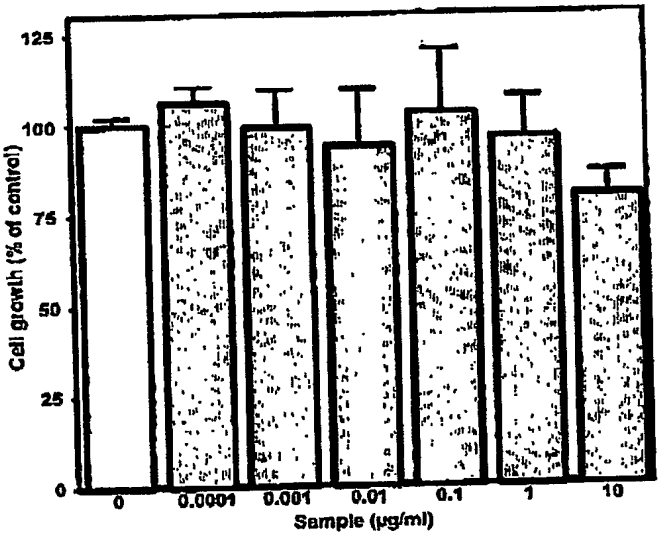


Figure 7. Effects of pomegranate fermented juice polyphenols (W) on proliferation of HUVEC (human umbilical vein endothelial cells) as measured by the MTT assay.

Plasminogen activator activity

Concentrations of W ranging from 10^{-4} to 10 micrograms per ml had no significant effect on plasminogen activator activity (data not shown).

Tubule formation by HUVEC

Figure 8 shows the effect of 10 micrograms per ml of different pomegranate fractions, or their combinations, on suppression of tubule formation from HUVEC. Note there is a suggestion of an additive effect from the combination of W and O. A similar pattern was noted at 5 micrograms per ml (not shown)

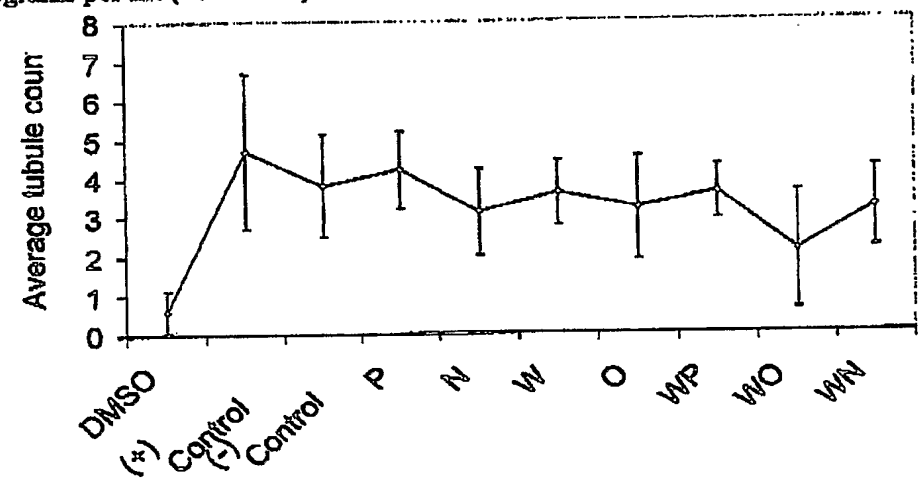
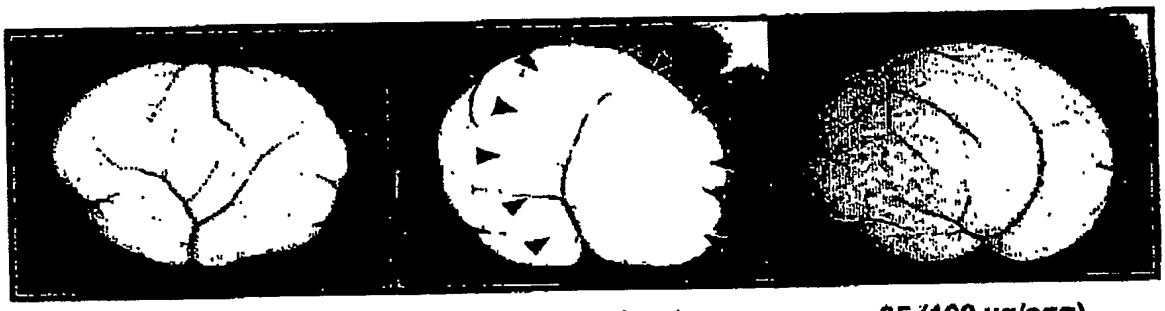


Figure 8. Effects of 10 micrograms per ml of selected pomegranate fractions and combinations on tubule formation in HUVEC that was stimulated by conditioned medium and soluble factors.

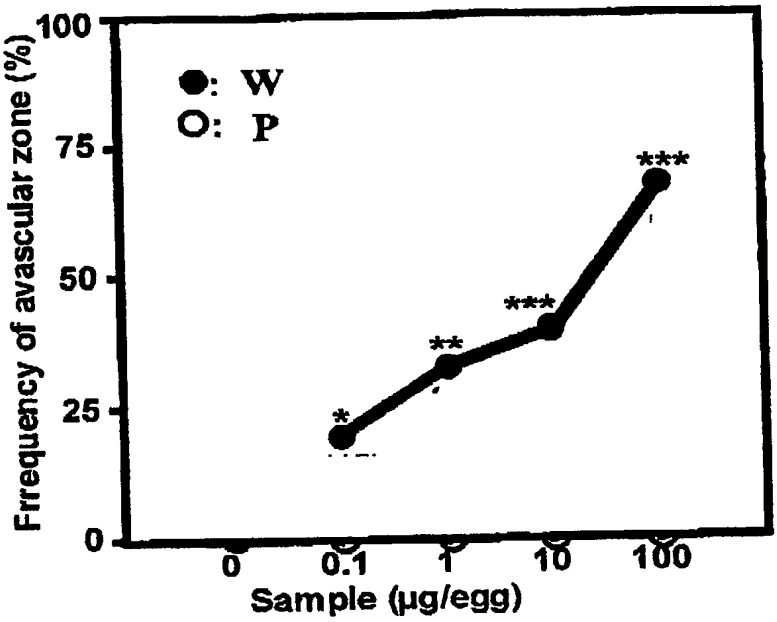
Suppression of Vascularization in Chick Embryo Chorioallantoic Membrane (CAM)

Figure 9a shows the suppression of vascularization in the CAM model by W, while P was without effect. Figure 9b demonstrates the same result graphically



W **P**

Figure 9a. Photomicrograph showing effect of pomegranate fraction W on neovascularization in chick embryo chorioallantoic membrane assay (CAM). Fraction P was without effect



*P<0.05
**P<0.01
***P<0.001

Figure 9b. Effect of pomegranate fermented juice polyphenols (W) on neovascularization in chick embryo chorioallantoic membrane assay (CAM). Pomegranate pericarp polyhenols (P) were without effect.

A simplified summary of the effects of all pomegranate extracts used in this study in all experimental settings is provided in Table 1.

Test														Fx
CAM angiogenesis inhibition *in vivo*	IL-4 upreg.			MIF upreg.			VEGF downreg.			HUVEC tubule formation inhibition	HUVEC proliferation inhibition	Amniotic fibrocyte proliferation inhibition	Myometrial fibrocyte proliferation inhibition	
	MCF-10-A	MB-MDA-231	MCF-7	MCF-10-A	MB-MDA-231	MCF-7	MCF-10-A	MB-MDA-231	MCF-7					
0	0	0	0	0	0	0	3	0	1	1	-	2	2	P
3	0	0	0	1	1	0	3	0	1	2	2	2	3	W
-	-	-	-	-	-	-	-	-	-	1	-	2	1	O
-	-	-	-	-	-	-	-	-	-	0	-	-	-	N
-	0	0	0	0	0	0	3	0	1	-	-	-	-	BOT
-	0	0	0	0	0	0	2	0	1	-	-	2	1	SEPO
-	0	0	0	0	2	0	3	0	2	-	-	-	-	SESCO
-	-	-	-	-	-	-	-	-	-	1	-	-	-	WP
-	-	-	-	-	-	-	-	-	-	2	-	-	-	WO
-	-	-	-	-	-	-	-	-	-	1	-	-	-	WN

Table 1: Summary of Effects of Pomegranate Fractions on Pharmacological Parameters Pertinent to Angiogenesis

Abbreviations: P = pomegranate pericarp polyphenols, W = pomegranate fermented juice polyphenols, O = pomegranate seed oil polyphenols N = non-sapoonified fraction of pomegranate seed oil, BOT = bottom fraction of pomegranate seed cake extract, SEPO = cold pressed pomegranate seed oil, SESCO = supercritical fluid extracted pomegranate seed oil, WP =1:1 mixture of pomegranate fermented juice and pericarp polyphenols, WO = 1:1 mixture of pomegranate fermented juice and seed oil polyphenols, WN = 1:1 mixture of pomegranate fermented juice polyphenols and non-saponified fraction of pomegranate seed oil,
HUVEC = human umbilical vein endothelial cells, VEGF = vascular endothelial growth factor, MIF = migration inhibitory factor, IL-4 = interleukin 4, CAM = chicken chorioallantoic membrane, MCF-7 = an estrogen receptor positive human breast cancer cell line, MB-MDA-231 = an estrogen receptor negative human breast cancer cell line, MCF-10A = an immortalized normal human breast epithelial cell line, downreg. = downregulation, upreg. = upregulation

Scale: (3) = strong effect, (2) = moderate effect, (1) = mild effect, (0) = no significant effect, (-) = not tested.

Discussion

Overall, this study helps to establish the potential of pomegranate fractions as potential adjuncts to both the chemoprevention and chemotherapy of breast cancer. The particularly striking suppression of VEGF in the most highly differentiated cell line, MCF-10A, is particularly suggestive of a role of these extracts in chemoprevention. That IL-4 was not affected in any cell line, helps to highlight the specificity of the effect of the extracts on VEGF.

Upregulation of MIF specifically in MDA-MB-231 is interesting in light of the seeming insensitivity of VEGF expression to the pomegranate fractions in this line. Thus, pomegranate fractions may possess antiangiogenic effects which involve downregulation of an angiogenic promoter (VEGF) in some types of cancer cells (MCF-7), and upregulation of angiogenic suppressors (MIF) in other cell types (MDA-MB-231). Such multiple cytokine or chemokine targeting by the fractions is a desirable attribute for anticancer therapy (Pavlakovic H, Havers W, Schweigerer L [26]).

Oxidative stress is an important trigger for angiogenesis (Maulik N, Das D. [27], Leek RD, Talks KL, Pezzella F et al. [28]), and the mechanism for this trigger likely involves NFkappaB (Sasaki H, Ray PS, Zhu L et al. [29]). Significantly, NFkappaB is important in mediating the antioxidant activity of pomegranate fermented juice polyphenols (Schubert SY, Neeman I, Resnick N. [30]). Therefor, the downregulation of VEGF and upregulation of MIF might possibly involve antioxidative mechanisms.

Pomegranate fractions, especially the seed oil, are known to possess estrogenic activity (Sharaf A, Nigm SAR 1964 [31]). Estrogens act on vascular endothelium (Cid MC, Schnaper HW, Kleinman HK. [32]) both through estrogen receptors (Gargett CE, Zaitseva M, Bucak K et al. [33]) and via NFkappaB (Galea E, Santizo R, Feinstein DL et al [34]. Thus, the effects here observed might also involve known patterns of estrogenic influence (White RE [35]). Specifically, the relative sensitivity of the estrogen receptor positive MCF-7 to downregulation of VEGF by the pomegranate fractions over the estrogen receptor negative MDA-MB-231 may partially be related to these mechanisms (Takei H, Lee ES, Jordan VC [36])

In addition, pomegranate seed oil consists largely of conjugated fatty acids similar to the conjugated linoleic acid (CLA) in bovine products. Recently, CLA has been shown to exert its antiangiogenic activity possibly through blockade of another angiogenic promotor found in fibroblasts, i.e., basic fibroblast growth factor (bFGF) (Moon, EJ, Lee YM, Kim KW; [37]). The punicic acid in pomegranate seed oil might act likewise.

Punicic acid inhibits prostaglandin formation (Nugteren DH, Christ-Hazelhof E. [38]). The inhibition of prostaglandin biosynthesis via cyclooxygenase results in downregulation of VEGF expression (Fukuda R, Kelly B, Semenza GL. [39]),and upregulation of MIF expression (Meyer-Siegler K. [40]). In that we observed the same pattern of VEGF downregulation and MIF upregulation from pomegranate fractions suggests inhibition of cyclooxygenase and / or other eicosanoid enzymes as a likely mechanism.

Generally, the present study highlights antiangiogenic potential for pomegranate extracts. We have shown this from suppression of fibroblast growth, suppression of HUVEC growth and suppression of HUVEC tubule formations. Angiogenic promoter molecules have been suppressed, and angiogenic inhibitory molecules promoted. Finally, inhibition of angiogenesis has been demonstrated *in vivo*. Undoubtedly, the results from the different pomegranate fractions, with their different chemistries, rely upon different and multiple mechanisms.

Pharmacological redundancy is a valuable and an important attribute of the pomegranate that may reinforce its applicability as an inhibitor of angiogenesis, and as an anti-cancer agent. The present study is in no way meant to be seen as comprehensive, as it leaves many gaps. Most notably, not all the fractions were tested in all of the models. However, in sum, there are sufficient indications to indicate anti-angiogenic potential of these extracts. In other settings (Lansky, EP, Jiang, WG, Mo, H et al [41]) we proved that the different fractions (e.g., P, W and SEPO or SESCO) acted synergistically to suppress prostate cancer proliferation and invasion, and also to suppress the activity of Phospholipase A2. Conceivably, such kind of synergistic interaction may also apply to the activities of these extracts against angiogenesis.

The extracts employed in the present study are components of a complex pomegranate drug in development for the prevention and / or treatment of breast and /or prostate cancer. The present preliminary studies lend credence to a more systematic examination of this agent in the context of angiogenesis.

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45

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Rapid Communication

Cooperative Suppression of PC-3 Human Prostate Cancer Cell Invasion by Pure Chemicals found in Anatomically Discrete Pomegranate (*Punica granatum*) Compartments

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SHORT TITLE: Cooperative Suppression of PC-3 Invasion

KEYWORDS: conjugated fatty acid, flavonoid, punicic acid, synergy

47

Abstract

BACKGROUND

Extracts from the seed, fermented juice and pericarps of *Punica granatum* fruits have been shown to potently inhibit human prostate cancer xenograft growth in mice, and proliferation and invasion of human prostate cancer cells *in vitro*. When these fractions were previously combined in equal amounts *in vitro*, markedly improved inhibition of PC-3 prostate cancer cell invasion across Matrigel compared to an equal amount of any of the single fractions was observed. To rule out the possibility that this effect was due to a single chemical which might have been present in all three fractions, we repeated the invasion experiment with pure chemicals known to occur exclusively in each of the three fractions.

METHODS

Human PC-3 prostate cancer cells were used as an *in vitro* model to assess invasion across a Matrigel™ artificial membrane. Invasion was stimulated with hepatocyte growth factor / scatter factor. Pure compounds known to be present in pomegranate peels (ellagic acid, luteolin), juice (caffeic acid) and seed oil (punicic acid) were purchased commercially and employed as potential inhibitors. The total dose was 4 µg / ml in each of 18 trials, whether compounds were tested singly, or in all possible equal combinations of two, three or four compounds. Invasion was visually assessed after 72 hours under inverted microscopy after staining and fixing the cells. The Kruskal-Wallis test was used to measure significance.

RESULTS

All four compounds individually inhibited invasion significantly, with the effect of caffeic acid much less than that of ellagic acid, punicic acid or luteolin. When any two compounds were combined, there were no significant benefits. However, combinations with three compounds did

result in statistically significant benefits, with the strongest effect from the equal combination of luteolin, punicic acid and caffeic acid. There was no added benefit from combining all four compounds.

CONCLUSION

These results highlight the anti-invasive properties of selected compounds naturally found in plants, and add credence to the hypothesis that the observed anti-invasive synergy between complex chemical fractions originating in anatomically discrete pomegranate compartments has a true multi-molecular basis.

INTRODUCTION

Pomegranate, *Punica granatum*, is a complex fruit containing structurally distinct cancer-suppressive chemicals originating in anatomically discrete anatomical compartments. These include, for example, the tannic acid, ellagic acid, from the peels [1], an exceptionally useful antioxidant [2] and proven inhibitor of both initiation and promotion of esophageal cancer *in vivo* [3] and prostate cancer cell gene expression *in vitro* [4]. Luteolin, a flavone with 58% the estrogenic activity of genistein [5], occurs in pomegranate peels along with the flavonols quercetin and kaempferol [6]. All three of these compounds potently arrest growth of PC-3 prostate cancer cells [7], but only luteolin inhibits production of the tumor necrosis factor, TNF-alpha [8], a promoter of ELAM-1, a cell adhesion molecule which plays a critical role in facilitating prostate cancer cell invasion [9]. Like pomegranate peels, pomegranate juice contains quercetin, but of the peels and juice, only pomegranate juice contains phenolic acids, such as caffeic acid [10], an inhibitor of tumor invasion across Matrigel [11].

Pomegranate seed oil, comprising 12-20% by weight of the seeds, consists of at least 65% punicic acid, a conjugated trienoic fatty acid [12]. Trienoic acids are more potent inhibitors of leukemia cell growth than conjugated dienoic acids [13], such as the known cancer preventive bovine derived compounds known collectively as conjugated linoleic acid (CLA) [14].

Our previous studies on pomegranate and prostate cancer have shown the pure seed oil and ethyl acetate extracts of the peel and fermented juice to inhibit cell growth and invasion, modulate gene expression, promote apoptosis and inhibit PC-3 xenografts in nude mice [15]. When the oil was combined equally with the juice and peel extracts, supra-additive suppression of prostate cancer cell growth, invasion and phospholipase A2

50

expression was observed compared to an equivalent dose of any one fraction [16]. In order to help rule out the possibility that the apparent supra-linear advantage of combining the different pomegranate fractions against prostate cancer cell invasion was in fact due to a single compound present in all three fractions, the present study was launched with pure chemicals believed to be more or less localized in individual pomegranate compartments.

MATERIALS AND METHODS

Phytochemicals

Small amounts of the putative anti-invasive agents, punicic acid (Larodan Chemicals, Malmo, Sweden) and caffeic acid, luteolin and ellagic acid (all from Sigma Aldrich, Rehovot, Israel) were sent to the experimental site in Cardiff, Wales in a coded, double-blind manner. Stock solutions were prepared in ethanol.

Invasion Assay

Our in vitro invasion assay was previously described [17]. Matrigel™ (Becton, Dickenson and Company, Franklin Lakes, NJ) was used to coat the 6.5 mm diameter polycarbonate membranes (pore size 8 μm) in the chambers of a 24-transwell system (Corning Costar Transwell, Cambridge, MA) at 50 μg / membrane. Following gel rehydration, 5×10^4 PC-3 human prostate cancer cells were added to each well. Hepatocyte growth factor / scatter factor (HGF) (Becton, Dickenson & Company, Franklin Lakes, NJ) was used at 40 ng / ml to induce invasion. The upper chamber contained punicic acid, luteolin, caffeic acid or ellagic acid, or a combination of two, three or all four of the chemicals at a fixed total concentration of 4 μg / ml. After 72 hr culture, invasive cells stuck to the lower surface were fixed and stained with crystal violet, and their number quantified under an inverted microscope and expressed as percentage of positive control.

Statistical Analysis

All preparations were tested in 5 separate assays. Ten separate assays were done for the positive control group (exposed to HGF only). The Kruskal-Wallis one-way nonparametric test was used to test for significant differences between the various preparations. A p value of < 0.05 was considered significant. If borderline significance was found, to increase the sensitivity we did paired comparisons between the assays in the order in

which they were done, and if all 5 pairs were higher in one group then that group was considered to have significantly less invasion ($p = 0.03$).

RESULTS

The mean value (cells invading) for the controls was 32.6 ± 10.7 and significantly higher than all preparations with single compounds (at least $p < 0.0196$) (Table 1, Figure 1). However caffeic acid had a significantly less effect when compared to the other single compounds (at least $p < 0.0088$). The various preparations with two components were not significantly better than the other single compounds (luteolin, punicic acid, ellagic acid), though a positive trend could be observed, as in the combination of punicic acid and ellagic acid. The best combination overall was luteolin, punicic acid and caffeic acid, which was also significantly better than the luteolin / punicic acid combination ($p = 0.03$, paired comparisons). Adding all 4 components together had no added benefit above the luteolin, punicic, caffeic combination, and there was even a trend for a reduced effect ($p = 0.09$).

CONCLUSION

The results here reinforce earlier work showing ellagic acid to potentiate the action of quercetin supra-additively in inducing apoptosis and suppressing proliferation in human leukemia cells [18]. In that case, a simple additive effect was an inadequate explanation for the extent of the increased overall anti-cancer activity.

Though single agents are the gold standard in pharmaceutical treatment, complex drugs are not completely unknown. One example is the estrogen complex known as Premarin, a mixture of ten estrogenic compounds derived from pregnant mare urine used for hormone replacement therapy. Another is Warfarin, a mixture of two racemic isomers used for preventing blood clots. From the point of view of multiple agents, synergistic effects resulting in overall therapeutic efficacy are also known in oncology [19].

Botanical extracts may contain combinations of bio-chemicals which collectively exert more potent activity than a single "active molecule" from the collection. This phenomenon is illustrated strikingly in the specific case of pomegranate. Caffeic acid, showing an overall much weaker anti-invasive effect than either luteolin or punicic acid, when combined with these two agents, results in a significantly greater overall effect than if only punicic acid and luteolin were combined alone. Appreciating this phenomenon may necessitate rethinking of optimum pharmaceutical strategies for treatment of cancer treatment in general, and for containment of prostate cancer in particular. Pomegranate, a generally recognized safe fruit with demonstrated chemopreventive activity in breast [20, 21] and skin cancers [22], and antiangiogenic [23] and differentiation-promoting [24] potential, is a putative source of a complex drug for treatment and / or prevention of prostate cancer.

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57

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Chemopreventive Effects of Pomegranate Seed Oil on Skin Tumor Development in CD_1 Mice

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ABSTRACT Pomegranate seed oil was investigated for possible skin cancer chemopreventive efficacy in mice. In the main experiment, two groups consisting each of 30, 4–5-week-old, female CD_1 mice were used. Both groups had skin cancer initiated with an initial topical exposure of 7,12-dimethylbenzanthracene and with biweekly promotion using 12-*O*-tetradecanoylphorbol 13-acetate (TPA). The experimental group was pretreated with 5% pomegranate seed oil prior to each TPA application. Tumor incidence, the number of mice containing at least one tumor, was 100% and 93%, and multiplicity, the average number of tumors per mouse, was 20.8 and 16.3 per mouse after 20 weeks of promotion in the control and pomegranate seed oil-treated groups, respectively ($P < .05$). In a second experiment, two groups each consisting of three CD_1 mice were used to assess the effect of pomegranate seed oil on TPA-stimulated ornithine decarboxylase (ODC) activity, an important event in skin cancer promotion. Each group received a single topical application of TPA, with the experimental group receiving a topical treatment 1 h prior with 5% pomegranate seed oil. The mice were killed 5 h later, and ODC activity was assessed by radiometric method. The experimental group showed a 17% reduction in ODC activity. Pomegranate seed oil (5%) significantly decreased ($P < .05$) tumor incidence, multiplicity, and TPA-induced ODC activity. Overall, the results highlight the potential of pomegranate seed oil as a safe and effective chemopreventive agent against skin cancer.

KEY WORDS: • *ornithine decarboxylase* • *Punica granatum* • *initiation* • *promotion* • *prostaglandins* • *punicic acid*

INTRODUCTION

SKIN CANCER is the most common type of cancer in the United States,[1] with more than a million reported cases[2] and 9,000 deaths per year.[3] Increasing incidence of these cancers due to constant exposure of skin to environmental carcinogens, including both chemical agents and ultraviolet radiation, provides a strong basis for chemoprevention with both synthetic and natural, and internal and topical, remedies.[6] Further, skin cancer chemoprevention is a useful model for cancer chemoprevention in general.[5]

Chemical and UVB radiation-induced skin carcinogenesis in murine skin and possibly human skin is a stepwise process of at least three distinct stages: initiation, promotion, and progression. Experimental initiation *in vivo* is accomplished by the topical application of a single dose of a skin carcinogen such as 7,12-dimethylbenzanthracene (DMBA), and is essentially irreversible. However, an initiation dose of carcinogen may not produce visible tumors, resulting only following prolonged and repeated application of a tumor promoter such as 12-*O*-tetradecanoylphorbol 13-acetate (TPA) to initiated skin.[6,7] Promoters like TPA induce ornithine decarboxylase (ODC), the rate-limiting enzyme in the synthesis of polyamines[8] and an important molecular target for skin cancer chemoprevention.[9] Other targets may also involve promotion, or initiation or progression events in the multistage process of neoplastic development.

Our previous work has highlighted the efficacy of topically applied natural products derived from onion and garlic oils,[10] and more recently sandalwood oil[11,12] and its constituent,[13] in preventing skin tumors in CD_1 and SENCAR mice. In the present work, we bring this experience to bear on the study of pomegranate seed oil as a potential skin cancer chemopreventive product.

Pomegranate fruit (*Punica granatum*) has been used worldwide as an item of diet and medicine for millenia, and has also been regarded as an important symbol in world religions and mythologies and of medicine itself.[14] We previously demonstrated potent antioxidant and prostaglandin-inhibitory activities for polyphenols extracted from pomegranate seed oil and pomegranate fermented juice,[15] as well as a wide range of human breast cancer suppressive properties *in vitro*, including promotion of apoptosis and inhibi-

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tion of proliferation and invasion by the seed oil, and inhibition of DMBA-initiated carcinogenesis in a mouse mammary organ culture (MMOC) by the fermented juice polyphenols.[16] We recently showed chemopreventive activity of the whole seed oil in the MMOC to be even stronger, weight per weight, than that of the purified fermented juice polyphenols.[17]

Pomegranate seed oil consists of >80% conjugated fatty acids, the most important of which is the octadecatrienoic acid, punicic acid. Punicic acid, like the <1% polyphenols in pomegranate seed oil, is an inhibitor of prostaglandin biosynthesis.[18] Punicic acid is also cytotoxic to mouse leukemia cells, possibility related to inhibition of lipid peroxidation.[19] Pomegranate is one of only about a half dozen plants known to contain conjugated fatty acids. A possible relationship between the relative botanical isolation of pomegranate and its singular chemistry and anticancer properties has been noted.[20]

The purpose of the present investigation was to study the chemopreventive effects of pomegranate seed oil on DMBA-initiated and TPA-promoted skin tumor development during the initiation and promotion phases in CD_1 mice. Further, the effects of pomegranate seed oil on weight gain and ODC activity in the experimental animals were also evaluated.

MATERIALS AND METHODS

Pomegranate seed oil

Pomegranate seed oil was provided by Rimonest Ltd. (Rimonest Ltd., Haifa, Israel; www.rimonest.com) from pomegranates of the "Wonderful" cultivar, organically grown at Kibbutz Sde Eliahu, Israel, in the year 2000. Seeds were separated from their juice sacs, washed in water, and dried in a convection current solar dryer. Oil extrusion was by "cold press" at 80°C, using a Type 40A electric screw press (Skeppsta Maskin, Orebro, Sweden). The oil was assayed by an independent laboratory (Mylnfield Research Services, Invergowrie, Dundee, Scotland) and shown to contain not less than 80% conjugated fatty acids as triglycerols, diglycerols, and monoglycerols.

Tumorogenesis protocol

The skin cancer protocol of Dwivedi *et al.*[13] was used. In brief, 4–6-week-old CD_1 mice were divided into two groups, each group containing 30 mice, as indicated in Table 1. The mice were kept in an environmentally controlled room with temperature, humidity, and light regulated. The backs of the mice were shaved carefully with an electric clipper to avoid cuts. The mice were allowed to rest for 2 days before carcinogenesis was initiated.

Table 1. Topical Treatments Received by the Mice in the Two Experiments

Group 1	100 μL of acetone
Group 2	100 μL of 5% pomegranate seed oil in acetone

Carcinogenesis was initiated with DMBA (200 nmol in 100 μL of acetone) applied topically. One week later, carcinogenesis was promoted with TPA (5 nmol in 100 μL of acetone), applied topically twice weekly. TPA treatment continued throughout the duration of the experiment (20 weeks). Mice in group 1 served as the control and were pretreated topically with 100 μL of acetone 1 h prior to each TPA application. Mice in group 2 were pretreated topically with 100 μL of 5% pomegranate seed oil in acetone 1 h prior to each TPA application. Tumor counts and group weights were taken on a weekly basis. Tumor incidence and multiplicity were calculated and analyzed statistically.

ODC assay

Mice were divided into two groups, each containing three mice. The backs of the mice were shaved carefully with an electric clipper to avoid cuts. Mice in group 1 received 100 μL of acetone before TPA (5 nmol in 100 μL of acetone) treatment topically. Mice in group 2 received 100 μL of 5% pomegranate seed oil in acetone, before topical TPA (5 nmol in 100 μL of acetone) treatment.

Mice were killed 5 h after the topical applications of TPA. The dorsal epidermis was removed and homogenized in phosphate buffer (pH 7.2) containing 0.1 m*M* pyridoxal phosphate and 0.1 m*M* EDTA. The homogenate was centrifuged at 105,000 *g* for 90 min and the supernatant collected and used for the ODC assay. The assay mixture in the main part of a Warburg flask was composed of 40 μL of phosphate buffer (pH 7.2), 25 μL of pyridoxal phosphate, 25 μL of dithiothreitol, 25 μL of EDTA, 10 μL of L-ornithine containing 0.5 μCi of DL[1-^{14}C]ornithine, and 200 μL of epidermal supernatant.

The center well of the Warburg flask contained 400 μL of ethanolamine and methoxyethanol used to absorb the $^{14}CO_2$ produced in the main compartment. After incubation at 37°C for 1 h, the reaction was stopped by the addition of 500 μL of citric acid. The mixture was stored in a dark place overnight to ensure complete absorption of $^{14}CO_2$ in the center well. The contents of the center well were transferred to a scintillation vial. The center well was washed with 0.5 mL of ethanol four times, and the wash also added to the scintillation vial, along with 10 mL of scintillation fluor. Radioactivity was counted with a Beckman LS6000SE liquid scintillation counter. The disintegrations per minute were quantified. Assessment of ODC activity was accomplished by measuring the production of $^{14}CO_2$ from DL-[1-^{14}C]ornithine.

Protein assay

Protein was assayed in the supernatant with a Bio-Rad Protein Assay Kit. A standard curve was obtained using bovine serum albumin. Absorbance values at 595 nm were determined using the spectrophotometer. Protein concentra-

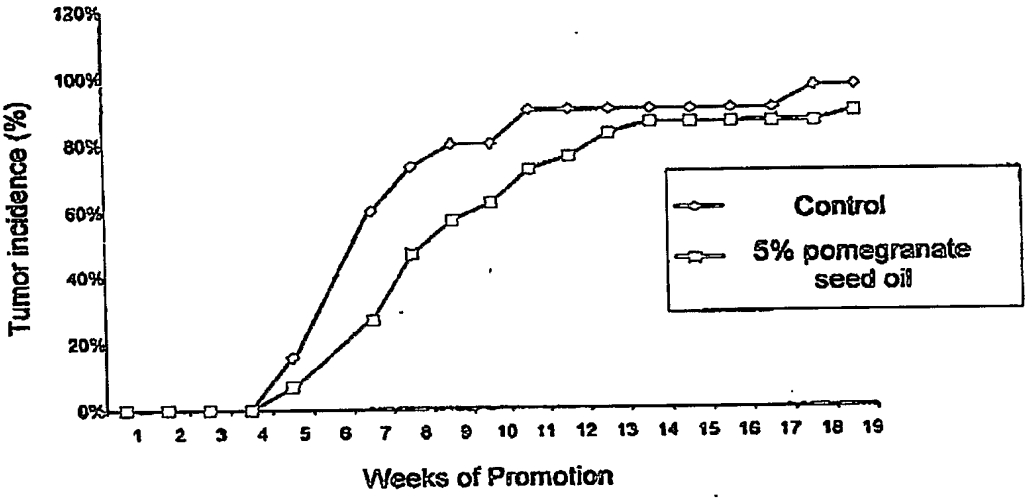

FIG. 1. The effects of pomegranate seed oil treatment on tumor incidence in CD_1 mice.

tions of the supernatant were extrapolated from the standard curve data.

Statistical analysis

The INSTAT software (GraphPad, San Diego, CA, U.S.A.) was used for the data analysis. χ^2 was used for the comparison of papilloma incidence and Student's t test for tumor multiplicity and ODC activity. Significance was considered at $P < .05$.

RESULTS

The effects of pomegranate seed oil treatment on the incidence of skin tumors in CD_1 mice are shown in Fig. 1. Skin tumors appeared in the sixth week of promotion after the initial DMBA application in the control and treated groups. Pomegranate seed oil treatment did not delay the appearance of tumors, but significantly decreased ($P < .05$) the rate at which the tumors developed. Skin tumor incidence after 20 weeks of promotion was 100% and 93% for

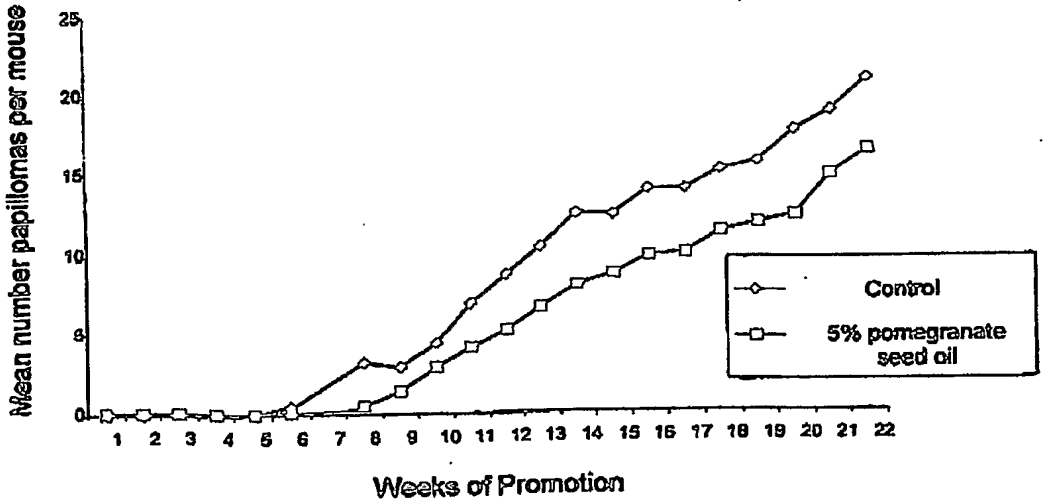

FIG. 2. The effects of pomegranate seed oil treatment on tumor multiplicity in CD_1 mice.

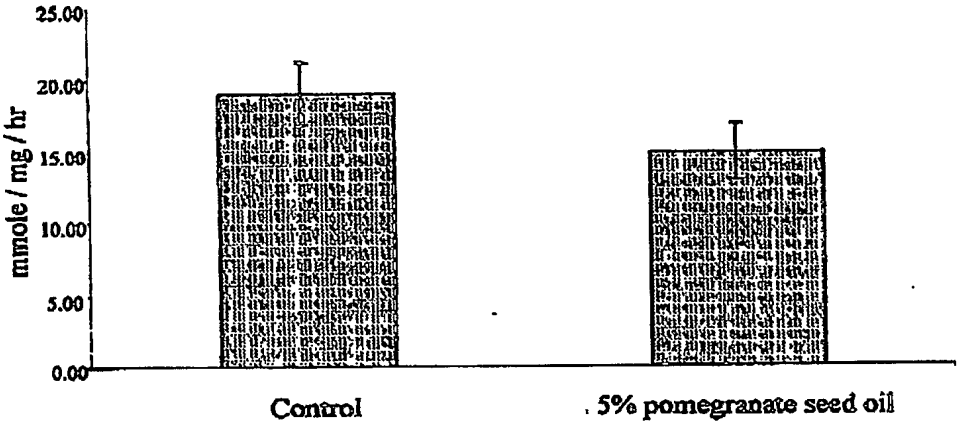

FIG. 3. The effects of pomegranate oil treatment on TPA-induced epidermal ODC activity in CD_1 mice. ODC activity is expressed as nmol of $^{14}CO_2$ produced/μg of protein/h. The 5% pomegranate seed oil (only) group did not have detectable ODC activity (data not shown).

the control and 5% pomegranate seed oil-treated groups, respectively.

The effects of pomegranate seed oil treatment on tumor multiplicity in CD_1 mice are shown in Fig. 2. Pomegranate seed oil treatment significantly decreased ($P < .05$) the tumor multiplicity throughout the 20 weeks of promotion. The mean number of tumors per mouse was 20.8 and 16.3 for the control and 5% pomegranate seed oil-treated groups, respectively.

Topical application of 5% pomegranate seed oil also significantly inhibited ($P < .05$) TPA-induced epidermal ODC activity. Fig. 3 illustrates the effects of pomegranate seed oil treatment on TPA-induced epidermal ODC activity. The ODC activity was 18.49 and 14.84 nmol of $^{14}CO_2$/mg/h in the control and 5% pomegranate seed oil-treated groups, respectively. The pomegranate seed oil group has significantly ($P < .05$) decreased ODC activity. Topical application of 5% pomegranate seed oil alone did not induce any epidermal ODC activity. Topical application of 5% pomegranate seed oil also did not have any effect on weight gain, as indicated in Fig. 4.

CONCLUSIONS

Pomegranate seed oil (5%) topical applications significantly decreased the incidence of skin tumor development,

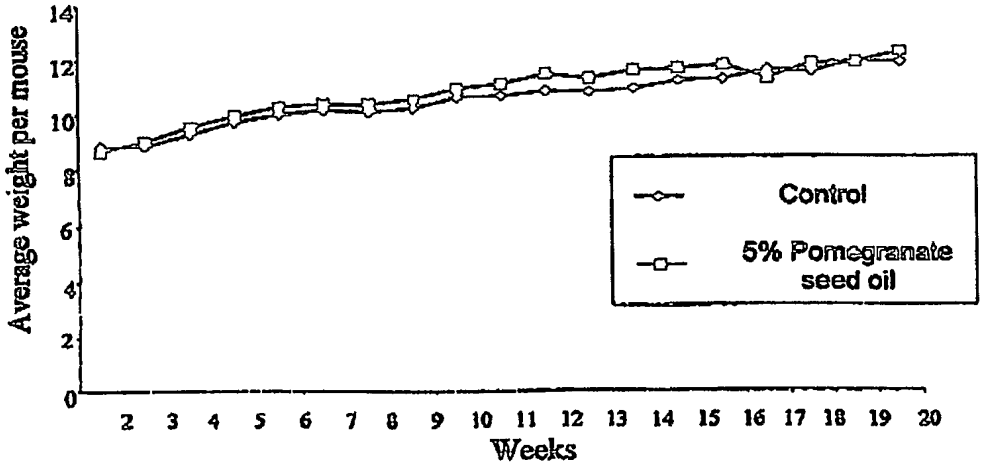

FIG. 4. The effects of pomegranate seed oil treatment on weight gain.

62

skin tumor multiplicity, and ornithine decarboxylase activity during 20 weeks of promotion. It is thus likely that the inhibition of ornithine decarboxylase by the pomegranate seed oil was at least partially responsible for the chemopreventive effect.

As noted, pomegranate seed oil is very rich in punicic acid, a known inhibitor of prostaglandin biosynthesis, specifically by inhibiting cyclooxygenase (Cox 1 and Cox 2) and lipoxygenase.[21] Pomegranate seed oil also inhibits the upstream eicosanoid enzyme, phospholipase A2, expressed by human prostate cancer cells.[22] That prostaglandins at very low concentrations promote ornithine decarboxylase[23] suggests that the inhibition of prostaglandin biosynthesis by pomegranate seed oil might also contribute to its inhibition of ornithine decarboxylase and, ultimately, to inhibition of skin cancer promotion.

Overall, pomegranate seed oil appears to be a benign natural product with potential as a topical chemopreventive agent against skin cancer. More in-depth investigations, including clinical studies, are warranted to evaluate this hypothesis further.

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Short Communication

"Pomegranate and the MMOC"

Breast Cancer Chemopreventive Properties of Pomegranate (*Punica Granatum*) Fruit Extracts in a Mouse Mammary Organ Culture (MMOC)

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64

Abstract.

We previously reported anticancer effects of pomegranate extracts in human breast cancer cells in vitro, and also chemopreventive activity of pomegranate fermented juice polyphenols (W) in a mouse mammary organ culture (MMOC). In the present study we decided to expand the MMOC investigations to also include an evaluation of the potential chemopreventive efficacy of a purified chromatographic peak of W (Peak B), and also of whole pomegranate seed oil. In brief, a mouse mammary organ culture was established according to a known method. For the first ten days of culture, the glands were treated with pomegranate fermented juice polyphenols (W), a high performance liquid chromatographic (HPLC) peak separated from W (Peak B), or pomegranate seed oil (Oil, and on day 3, exposed to the carcinogen 7,12-dimethylbenz[a]anthracene (DMBA), and for ten days treated with the putative pomegranate chemopreventive. The glands were subsequently harvested and tumors counted by visual inspection. While W effected a 42% reduction in the number of lesions compared to control, Peak B and pomegranate seed oil each effected an 87% reduction. The results highlight enhanced breast cancer preventive potential both for the purified compound Peak B and for pomegranate seed oil, both greater than that previously reported for pomegranate fermented juice polyphenols.

KEYWORDS: breast cancer, CLA, conjugated fatty acid, conjugated triene, flavonoid, menopause, polyphenol, pomegranate seed oil, punicic acid

Introduction. Pomegranate (Punica granatum) is an ancient fruit with extensive regard both as a symbol and as an article of medicine (Langley, 2000). Our previous work with pomegranate fractions demonstrated antioxidant and eicosanoid enzyme inhibition (Schubert, Lansky and Neeman, 1999), suppression of breast cancer growth and invasion, and inhibition of breast cancer carcinogenesis (Kim et al, 2002), inhibition of skin cancer carcinogenesis (Hora et al, 2003), and inhibition of angiogenesis (Toi et al, 2003). Additional pharmacological actions have also been recorded, especially that owing to both the prevention and reversal of atherosclerotic lesions (Aviram et al, 2002).

In earlier studies we showed that the effects of pomegranate fruit may be largely due to the presence of selective polyphenols. However selective role of each polyphenol in MMOC has not been investigated. Previously we showed that the fruit extract (W) exhibits chemopreventive activity in mouse mammary gland organ cultures (Kim et al 2002). However the effect was not dramatic. Here we intended to compare the activity of the fruit extract (W) with that of isolated HPLC peaks in mammary lesion formation. We selected one of these isolates, designated as peak B and compared its activity with that of the parent fruit extract. Pomegranate seed oil is rich (~80%) in conjugated fatty acids and contains small amounts of other physiologically active compounds such as gamma-tocopherol, campestrol, sigmasterol, sitosterol, estrone and alpha-estradiol (Table 1). Punicic acid alone, which comprises 65% of the whole oil, is a significant inhibitor of prostaglandin biosynthesis (Nugteren and Christ-Hazelhof 1987). Thus pomegranate seed oil, which we found to be a potent inhibitor of breast cancer cell invasion in very small doses (3 μg / ml) (Kim et al 2002) may also have broader potential as an anti-inflammatory agent. We compared here the activity of pomegranate seed oil with that of the fermented juice extract (W) and

HPLC isolate (Peak B). The present study is the first to examine the potential chemopreventive utility of a possibly novel pomegranate compound (Peak B), and in addition, expands the known potential chemopreventive applications for pomegranate seed oil.

Materials and Methods.

Pomegranate extracts.

Organically grown pomegranates of the "Wonderful" cultivar from the 2000 crop of Kibbutz Sde Eliahu, Israel were utilized to produce all the putative chemopreventive agents. Pomegranate seed oil was prepared by cold-press at 80° C using an electric seed pressing machine (40A, Skeppsta Maskin, Orebro, Sweden) from washed and dried seeds from which the juice had been previously removed. Pomegranate fermented juice polyphenols (W) were obtained by layering concentrated fermented pomegranate juice over ethyl acetate overnight (12 hours). In the morning, the ethyl acetate layer was separated with a separation funnel, and evaporated under nitrogen gas to yield the polyphenols (W). Peak B was obtained from a specific peak on a high performance liquid chromatogram using preparative HPLC technique. A schematic diagram for the extraction and efficacy in MMOC assay is summarized in Figure 1.

Mouse Mammary Organ Culture

A murine mammary gland organ culture was established according to a known method (Mehta et al 1991, Mehta 2000). The study was approved by the University of Illinois Animal Review Board and performed in accordance with institutional guidelines. Briefly, young, virgin BALB/c female mice, 3-4 weeks of age (Charles River Laboratories, Wilmington, MA, USA) were pretreated for 9 days with 17-β-

estradiol (1 μg in 0.1 mL of saline / animal) and progesterone (1mg in 0.1 mL of saline / animal). They were subsequently killed by cervical dislocation, and the thoracic pair of mammary glands removed, placed on silk rafts, and incubated for 10 days in serum-free Waymouth MB752 medium (Life Technologics, Inc, Gaithersburg, MD, USA) containing the following growth-promoting hormones: insulin (5 μg / mL), prolactin (5 μg / mL), aldosterone (1 μg / mL), and hydrocortisone (1 μg / mL). The carcinogen 7,12-dimethylbenz[a]anthracene (DMBA) at a dose of 2 μg / mL in DMSO was added to the medium on day 3 for a duration of 24 hours to induce mammary lesions. The mammary glands were incubated for an additional 14 days with medium containing only insulin. This procedure allowed the normal glands to undergo structural regression in which all the normal alveolar structures were disintegrated. But alveolar lesions in the carcinogen-treated glands behave differently, namely, they acquire altered hormone responsiveness and continue to grow. Between 11-15 mammary glands per group were employed in four different groups: a control, and those treated with either fermented juice polyphenols (W), purified fraction Peak B, or pomegranate seed oil (Oil). The three agents were individually included in the media during the first 10 days of in vitro culture to determine if they lowered the incidence of formation of mammary lesions. Throughout the culture period, the glands were maintained at 37 ° C in a 95% O2 and 5% CO2 atmosphere. At the end of the culture period, the glands were fixed in formalin, stained in alum-carmine solution, and evaluated for presence or absence of mammary lesions. The multiplicity of the lesions (number of lesions per gland) was not scored for the present study. Previous studies from our laboratory have shown that in using 15 glands per group the incidence varies from 53% to 100% of glands

with lesions. All hormones and chemicals were purchased from Sigma Chemical Company (USA). The procedure is diagrammatically shown in Figure 2.

Statistical analysis. Probabilities for the relative differences between the experimental groups were determined with the Chi-square test, with $p < 0.05$ considered significant. In general, ssuming 60% incidence of lesions in control glands, and 60% suppression by a chemopreventive agent in relation to control glands results in statistical significant inhibition using Chi square analysis.

Results. Previous studies with mouse mammary gland organ culture have shown that treatment with DMBA (2μg/ml medium) induces precancerous mammary lesions in the gland with the frequency of 60-100%. Consistent with these results, in the present experiment 73% (11/15) of the control glands had mammary lesions. The phenolic extract of the fermented fruits (W) showed mammary lesion incidence of 37% (6/13 glands with lesions) resulting in 38% inhibition of mammary lesions formation. However the effect was not statistically significant. On the other hand, treatment with both pomegranate seed oil and the purified HPLC peak (Peak B) derived from a phenolic fraction of fermented pomegranate juice (W) demonstrated significantly greater chemopreventive potential than W. As compared to 38% inhibition of lesion formation by W, there was 75-90% suppression by peak B and seed-oil ($p<0.05$). The chemopreventive potential of the seed oil was greater at low dose (1 μg / mL) than normal dose (10 μg / mL). The experiment was repeated and the same result obtained. These results are shown in Figure 3.

Discussion: The MMOC provides at least a 75% predictive accuracy of in *vivo* carcinogenesis (Mehta 2000), here strongly suggesting a chemopreventive role for pomegranate fractions in breast cancer. The success of the Peak B in this model demonstrates the existence of a fraction possibly representing a pure compound within the fermented juice phenolic mixture possessing greater chemopreventive utility than the parent extract. Efforts to further purify and identify this compound are in progress.

Especially striking is the chemopreventive activity of the pomegranate seed oil, which appears to peak at very low doses. In this study, at least, this oil demonstrated chemopreventive activity equivalent to that of the semi-purified compound from the juice fraction. Pomegranate oil consists largely of punicic acid, a conjugated 18-carbon trienoic acid. The punicic acid and its isomers constitute approximately 80% of the oil (Schubert, Lansky and Neeman 1999). Trienoic acids such as punicic acid exert cancer suppressive utility greater than that well established for dienoic acids, such as the bovine-derived conjugated linoleic acid (CLA) (Igarashi and Miyazawa 2000). The chemopreventive effect of pomegranate seed oil likely owes much to punicic acid, through inhibition of eicosanoid metabolism leading to prostaglandin biosynthesis has also been demonstrated for the polyphenol component (<1%) of the oil alone.

This preliminary report clearly suggests that the both the pomegranate seed oil and fermented fruit extracts exhibit chemopreventive activity and the activity of the fruit extract may in part be due to the phenolic compound present in peak B of the HPLC isolate, whereas the efficacy of seed oil may be due to the presence of punicic acid present in the oil. Challenges lie ahead to design chemopreventive protocols to get the most out of the potential inherent in pomegranate. Pomegranate juice alone,

though helpful, obviously misses the great potential of pomegranate seed oil. A clue to designing effective pomegranate chemopreventives might be taken from the painting Proserpina (Fig 4) by the 19th century Italian painter Dante Gabriel Rossetti, where the maiden mystery looks back at us having apparently just taken a bite out of a whole pomegranate, rind, seeds and all! While pursuit of pure active compounds is the traditional occupation of drug discovery, the best strategy might yet be one which makes use of different parts of the fruit, all of which have demonstrated chemopreventive activity concealed within.

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Table 1

Analysis of Pomegranate Seed Oil used in this Study

Fatty acid profile (%)	mg/g	%
16:0 palmitic	3.8	9.6
18:0 stearic	2.2	6.0
18:1 oleic	6.8	18.5
18:2 linoleic	6.5	17.4
18:3 Y-linolenic	0.6	1.2
18:3 conjugated trienes	65.3	175.6
20:0 icosanoic	0.4	1.1
20:1 icosenoic	0.6	1.7
22:0 docosanoic	1.3	3.4
22:1 docosenoic	2.9	8.0
22:5 docosapentaenoic	0.3	1.1
24:0 tetracosanoic	1.4	3.0
24:1 tetracosenoic	0.5	1.1
minor components	7.6	

The relative proportions of phospholipid, monoacylglycerol, diacylglycerol and triacylglycerol are quantified as C18 fatty acids containing species and the wax ester as the C46 wax ester species.

Component	%
Phospholipid	3
Monoacylglycerol	1
Diacylglycerol	8
Triacylglycerol	80
Wax Esters	7

Minor components:

Sitosterol	0.6%
Campesterol	0.1%
Stigmasterol	0.01%)
Gamma-tocopherol	0.3%)
17 alpha estradiol	0.3%
%	
Other	~01.6%

Analysis was performed on a capillary column using gas chromatography with mass spectrometry. Balances and thermometers are calibrated regularly. All products are prepared according to master documents that assure manufacture according to validated methods. Batch records document raw material traceability and production and testing history for each lot manufactured.

34

Figure 1 Extraction procedure for identifying and evaluating pomegranate extracts and isolates.

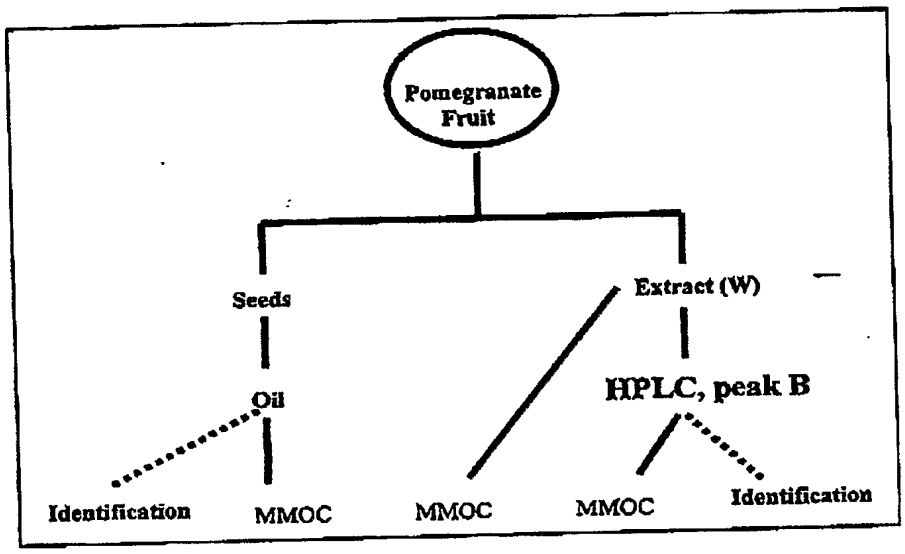

Figure 2. Experimental design to induce mammary lesions in the gland its prevention by pomegranate isolates

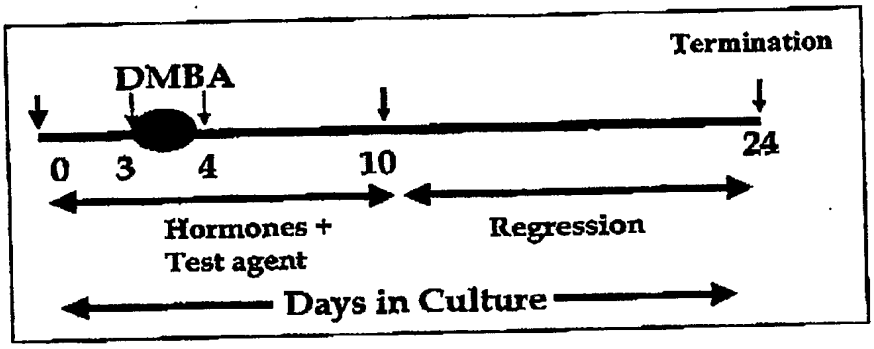

Figure 3. Effect of different pomegranate fractions on suppression of tumorigenesis in a Mouse Mammary Organ Culture (MMOC) model. W = pomegranate fermented juice polyphenols, Peak B = semi-purified peak separated from W by preparative high performance liquid chromatography (HPLC), Oil = cold-pressed pomegranate seed oil. Significance: $p < 0.05$ in all cases by chi-square analysis. N = 11-15 glands per group.

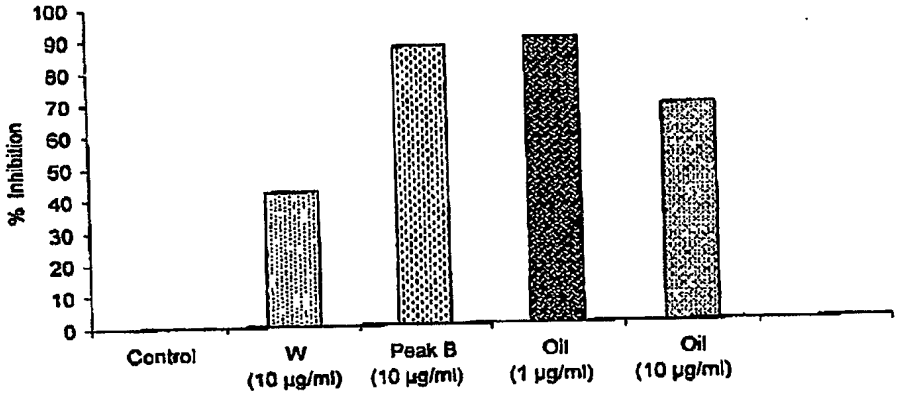

Figure 4. *Proserpina,* Dante Gabriel Rossetti, 1874.

Rapid Communication

Cooperative Suppression of PC-3 Human Prostate Cancer Cell Invasion by Pure Chemicals found in Anatomically Discrete Pomegranate (*Punica granatum*) Compartments

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SHORT TITLE: Cooperative Suppression of PC-3 Invasion

KEYWORDS: conjugated fatty acid, flavonoid, punicic acid, synergy

79

Abstract

BACKGROUND

Anatomically discrete extracts of *Punica granatum* were previously shown to cooperatively inhibit prostate cancer cell invasion *in vitro*. Here we similarly studied pure compounds from those extracts.

METHODS

Luteolin, ellagic acid and caffeic acid from the aqueous pomegranate phase (juice, peels) and punicic acid from pomegranate seed oil were tested as putative inhibitors of HGF-stimulated invasion of human PC-3 prostate cancer cells across Matrigel™. The total dose was 4 μg / ml in each of 18 trials, whether one, two, three or four compounds were employed. Invasion was visually assessed after 72 hours. The Kruskal-Wallis test was used to test for statistically significant differences.

RESULTS

All four compounds individually inhibited invasion significantly, and the equal combination of luteolin, punicic acid and caffeic acid resulted in significant supra-additive enhancement.

CONCLUSION

The results suggest that the observed anti-invasive synergy of discrete pomegranate extracts has a molecular basis.

INTRODUCTION

Pomegranate, *Punica granatum*, contains structurally distinct prostate cancer-suppressive chemicals. These include, from the peels and juice, ellagic acid [1], luteolin [2, 3], and caffeic acid [4]. Pomegranate seed oil is 60% punicic acid [5] a trienoic conjugated fatty acid with greater antiproliferative activity in leukemia cells than dienoic conjugated linoleic acid (CLA) [6].

Pomegranate extracts inhibit prostate cancer cell growth and invasion, modulate gene expression, promote apoptosis and inhibit PC-3 xenografts in nude mice [7]. Combinations of these different extracts supra-additively suppress prostate cancer cell growth, invasion and phospholipase A2 expression [8]. To explore the mechanism of this phenomenon, we tested representative pure compounds from these extracts in the *in vitro* invasion assay.

MATERIALS AND METHODS

Phytochemicals

Small amounts of the putative anti-invasive agents, punicic acid (Larodan Chemicals, Malmo, Sweden) and caffeic acid, luteolin and ellagic acid (Sigma Aldrich, Rehovot, Israel) were packaged and tested by two teams in a coded, double-blind manner. Stock solutions were prepared in ethanol (final concentration < 1%).

Invasion Assay

Our *in vitro* assay using Matrigel™ (Becton, Dickenson and Company, Franklin Lakes, NJ) was previously described [9]. Hepatocyte growth factor / scatter factor (HGF) (Becton, Dickenson & Company, Franklin Lakes, NJ) was used (40 ng / ml) to induce invasion. The upper chamber contained punicic acid, luteolin, caffeic acid or ellagic acid, or a combination of two, three or all four of the chemicals at a fixed total concentration of 4 µg / ml. After 72 hr culture, invasive cells stuck to the lower surface were fixed and stained with crystal violet, and their number quantified under an inverted microscope and expressed as percentage of positive control.

Statistical Analysis

All preparations were tested in 5 separate assays. Ten separate assays were done for the positive control group (exposed to HGF only). The Kruskal-Wallis one-way nonparametric test was used to test for significant differences between the various preparations. A p value of < 0.05 was considered significant. If borderline significance was found, to increase the sensitivity we did paired comparisons between the assays in the order in which they were done, and if all 5 pairs were lower in one group, then that group was considered to have significantly less invasion ($p = 0.03$).

RESULTS

The mean value (cells invading) for the controls (with HGF alone) was 32.6 ± 10.7 and significantly higher than all preparations with single compounds (at least $p < 0.0196$) (Figure 1). Caffeic acid had a significantly less effect when compared to the other single compounds (at least $p < 0.0088$). The various preparations with two components were not significantly better than the other single compounds (luteolin, punicic acid, ellagic acid), though positive trends could be observed. The best combination was luteolin, punicic acid and caffeic acid, which was also significantly better than the luteolin / punicic acid combination ($p = 0.03$, paired comparisons). Adding all 4 components together had no added benefit above the luteolin, punicic, caffeic combination, and there was even a trend for a reduced effect ($p = 0.09$).

CONCLUSION

Though single agents are the gold standard in pharmaceutical treatment, complex drugs are not completely unknown. One example is the estrogen complex known as Premarin, a mixture of ten estrogenic compounds derived from pregnant mare urine used for hormone replacement therapy. Another is Warfarin, a mixture of two racemic isomers used for preventing blood clots. Multiple agents are used synergistically in oncology [10].

Pomegranate contains pharmacologically active chemicals in its aqueous and oily compartments which contribute to its anti-cancer effects [11, 12, 13], including promotion of differentiation [14] and suppression of angiogenesis [15]. The present study suggests that these chemicals, when combined, may account for the previously observed synergy between pomegranate fractions in suppressing prostate cancer invasion *in vitro*, and could provide the basis of a complex drug for containing prostate cancer.

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Figure Legend

Fig. 1 **Effect of Pure Chemicals from Pomegranate and their Combinations on Human PC-3 Prostate Cancer Cell Invasion *In Vitro*.** L = luteolin, P = punicic acid, C = caffeic acid, E = ellagic acid The combination of luteolin, caffeic acid and punicic acid was significantly better than punicic acid alone ($p < 0.0196$), and of punicic acid and luteolin alone ($p = 0.03$). The total dose of chemical(s) was 4 µg / ml in all cases.

What is claimed:

1. A pomegranate derived product with anti-angiogenic activity essentially as described herein.

2. A method of preventing or retarding angiogenesis comprising administering a product of claim 1 to a subject.

3. A pharmaceutical composition comprising, as an active ingredient a product of claim 1.

4. An article of manufacture comprising a pharmaceutical composition according to claim 3 and labeled as having anti-angiogenic activity.

5. A juice drink consisting of emulsifier, pomegranate seed oil and fermented and/or unfermented pomegranate juice, as a general health tonic.

6. .An additive to wine for antioxidant benefit consisting of an alcoholic extract of whole pomegranates, the extract consisting of pure distilled pomegranate spirits removed from fermented pomegranate and whole pomegranates.

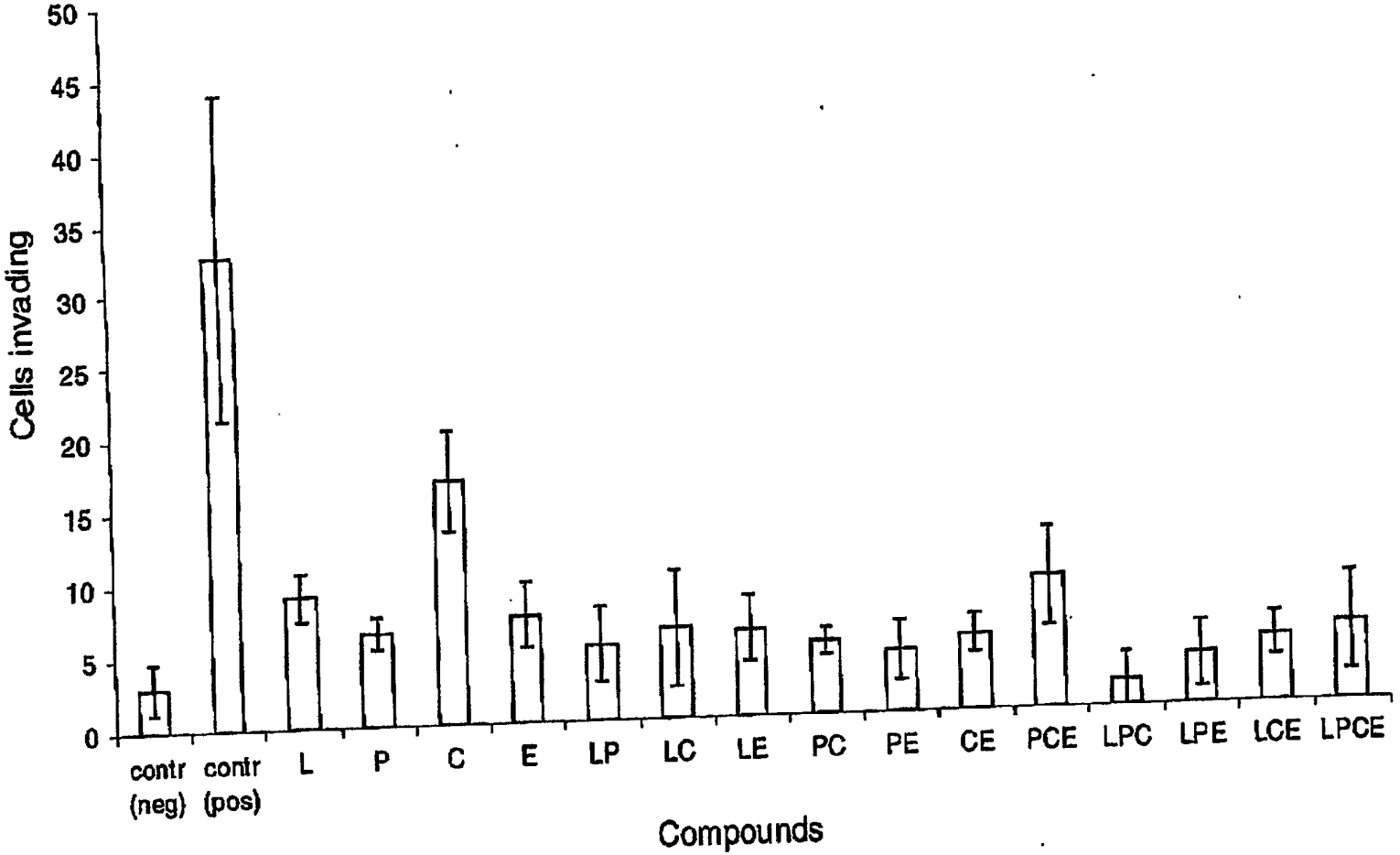
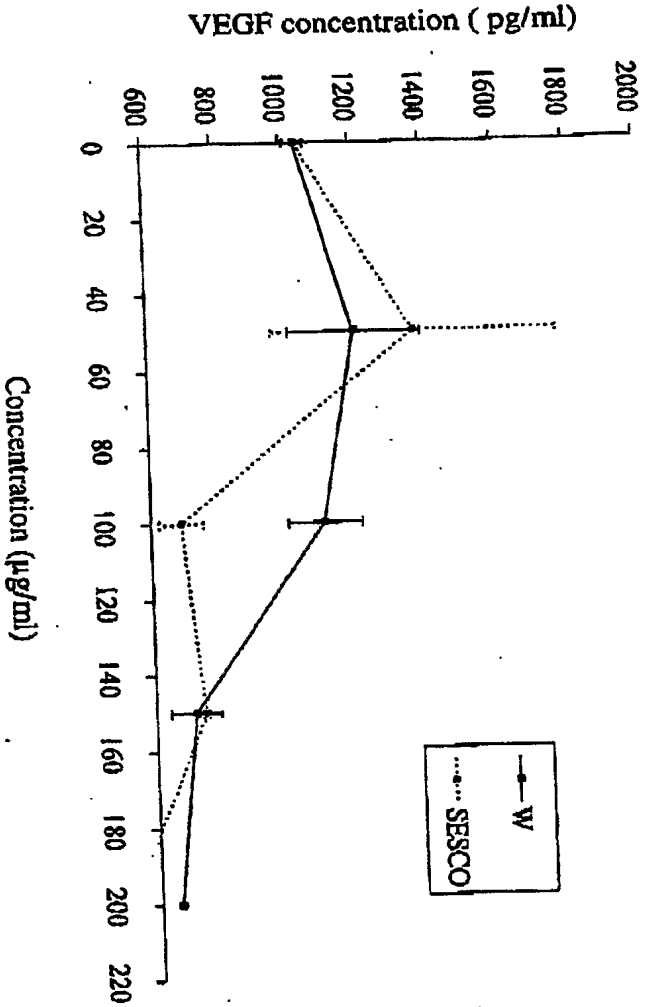
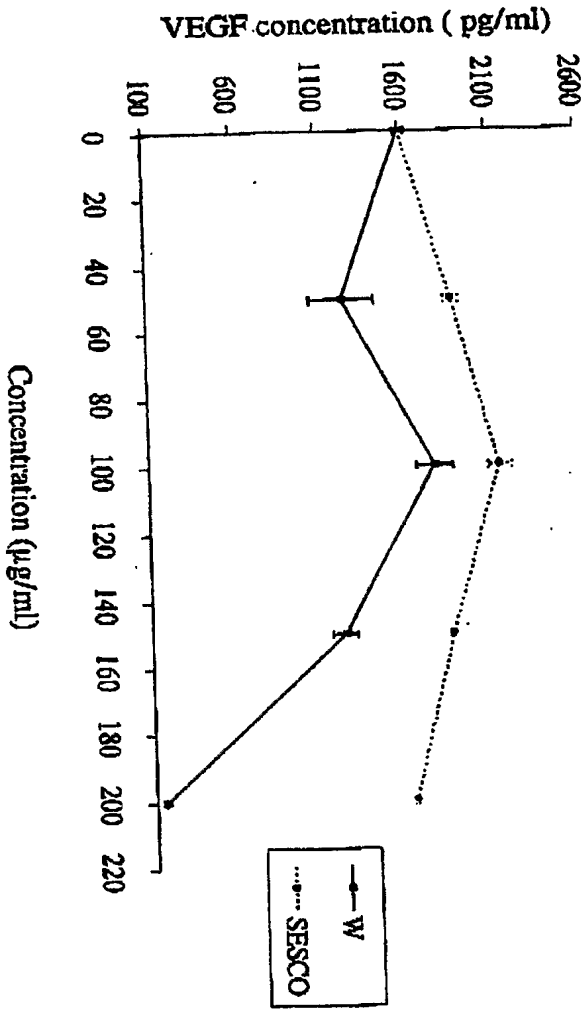


Figure 1. Suppression of Invasion by Pure Chemicals and Combinations

L = luteolin, P = punicic acid, C = caffeic acid, E = ellagic acid





5

Table 1. Effects of pure chemicals on PC-3 Invasion

Single Agents			Doublets			Triplets			Quadruplet		
	X	s		X	s		X	S		X	s
L	9	1.6	L P	5.2	2.6	P C E	9.0	3.4	L P C E	5.4	3.4
P	6.4	1.8	L C	6.2	4.0	L P C	1.8	1.8			
C	16.8	3.4	L E	6.0	2.2	L P E	3.4	2.3			
E	7.4	2.3	P C	5.0	1.0						
c+	33	10.7	P E	4.2	2.0						
c-	3	1.7	C E	5.2	1.3						

L= luteolin, P = punicic acid, C = caffeic acid, E = ellagic acid,

c+ = positive control, c- = negative control,

X = mean number of invading cells, s = standard deviation